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ENDOCRINOLOGY

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IDENTIFICATION OF THREE TRANSFORMATION PRODUCTS AFTER AN ADRENAL PERFUSION WITH EPIANDROSTERONE[1,2]

ANDRE S. MEYER, ORVILLE G. RODGERS AND GREGORY PINCUS

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THE technique of bovine adrenal perfusion has been utilized in the study of the metabolism of steroids (cf. Hechter *et al.*, 1951). Detailed reports of perfusions with various compounds such as desoxycorticosterone and 17-hydroxy-desoxycorticosterone (Levy *et al.*, 1953) and cortisone (Meyer, 1953a) have been published. In addition several 17-ketosteroids have been perfused under similar conditions. These include androsterone (Hechter *et al.*, 1950), Δ^4-androstene-3,17-dione (Jeanloz *et al.*, 1953), and dehydroepiandrosterone (Meyer *et al.*, 1953). Epiandrosterone (androstane-3β-ol-17-one) was selected for study as the next steroid in this series. This communication is a report of this perfusion and the products isolated from it. Approximately 33% unchanged epiandrosterone (II) was recovered. In addition about 10% androstane-3,17-dione (I) was obtained. 11β-Hydroxylation was also noted, but occurred in a rather limited degree; androstane-11β-ol-3,17-dione (III) and androstane-3β,11β-diol-17-one (IV) were isolated at only 3 and 1% levels. It is believed that other transformation products were present in minor quantities, but could not be detected with the techniques used.

EXPERIMENTAL[3]

Starting material.—Epiandrosterone was obtained in 97% yield from the hydrolysis of its acetate[4] with potassium carbonate in aqueous methanol

Received for publication February 14, 1953.

[1] This investigation was supported by a grant from G. D. Searle and Company, Chicago, Illinois.

[2] This is paper VII in the series: Chemical Transformation of Steroids by Adrenal Perfusion. For Paper VI, see Meyer (1953b).

[3] Melting points were observed on a Fisher-Johns hot-stage through a microscope and are corrected (accuracy $\pm 2°$).

at 40° (cf. v. Euw and Reichstein, 1942). Crystallized from acetone-hexane or benzene-hexane it had a m.p. 177–178° and $[\alpha]_D^{27}+88°\pm2°$ (c, 0.794 in chloroform). These physical data are in good agreement with those in the literature (Selye, 1943).

Perfusion.[5]—The perfusion was carried out according to the technique of Hechter *et al.* (1953) detailed in paper I of this series. A solution of 600

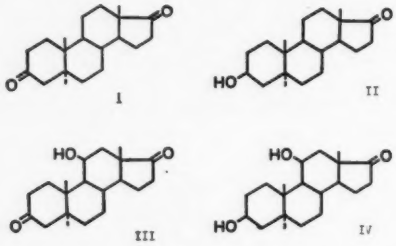

FIG. 1

mg. of epiandrosterone in 10 ml. of propylene glycol was combined with 6 L. of citrated bovine blood containing antibiotics. This mixture was circulated via venous entry through four lacerated cow adrenals placed in parallel. The perfusion was carried out under an O_2/CO_2 atmosphere for $3\frac{1}{2}$ hrs. at 37°. The blood was passed approximately 5 times through the glands.

Extraction and fractionation of the steroids from the perfusate.—The perfusate was processed by a method described previously (Meyer *et al.*, 1953) utilizing the charcoal procedure (Levy *et al.*, 1953). Two portions of Darco G-60 (160 and 80 gm.) were employed for adsorption of the steroids from the perfusate over contact periods totalling 48 hrs. The blood was kept at 4° throughout the entire time. Elution of the adsorbed steroids was undertaken with the azeotropic mixture of benzene-methanol, followed by the usual series of solvents. While this mixture extracted the bulk of the material, it proved less desirable than acetone or methylene chloride used in previous work, since more nonsteroidal matter was also eluted. A total of 3.4 gm. of a brown syrup was obtained and fractionated on a silica gel column (Table 1).

ISOLATION AND IDENTIFICATION OF COMPOUNDS

Androstane-3,17-dione (I).—The 69 mg. of a low melting material from

[4] The androstane-3β-ol-17-one-acetate was obtained from Dr. H. Levy (Levy & Jacobsen, 1947).

[5] The perfusion was carried out by Dr. V. Schenker.

TABLE 1. SILICA GEL CHROMATOGRAPHY OF THE PERFUSATE EXTRACT

3.4 gm. extract, eluted from the charcoal, were dissolved in benzene and placed on 95 gm. silica gel (1.8×56 cm.). 200 ml. portions of solvent were used per fraction

Fractions	Elutions	Dried eluate		After first crystallization		Compounds eventually identified
		Weight in mg.	Description	Weight in mg.	m.p.	
1– 4	Benzene, benzene-ether up to 4:1 (by volume)	230	waxy substance			
5	Benzene-ether 3:1	131	waxy substance	69	50–125°	I
6	Benzene-ether 3:1	29	syrup	0.3	>300°	
7– 9	Benzene-ether 2:1	252	m.p. 167–175°	180	172–175°	II
10–12	Benzene-ether 1:1 and 1:2	109	m.p. 100–140°	15	180–210°	II, III
13–17	Benzene-ether up to 1:9	83	syrup	16	>300°	
18–24	Ether, ether-ethyl acetate 19:1, 9:1	53	syrup	15	175–230° 220–231°	IV, V
25–30	Ether-ethyl acetate up to 1:1	73	partially cryst.	20	185–191°	V
31–34	Ethyl acetate, chloroform	224	dark syrup			
35–37	Methanol, methanol-glacial acetic acid 49:1	375	dark syrup			

fraction 5 of the chromatography yielded after four recrystallizations from ether-pentane and sublimation in a high vacuum, 20 mg. of platelets with a m.p. 134–136° and $[\alpha]_D^{24}+107°\pm3°$ (c, 0.918 in chloroform). A sample of commercial androstane-3,17-dione sublimed and crystallized in the same manner was found to melt at 132–134°, $[\alpha]_D^{27}+112°\pm3°$ (c, 0.537 in chloroform). Both substances were converted into the bisdinitrophenylhydrazone by the procedure of Liebermann *et al.* (1948) and melted at 254–257°. Mixed m.p. determinations of the free compounds and of the phenylhydrazones did not show a depression.

Epiandrosterone (II).—The 180 mg. of crystals obtained in fractions 7 to 9 were recrystallized from methanol-water and gave a m.p. 175–177°, $[\alpha]_D^{28}+89°\pm3°$ (chloroform). A mixture with a sample of the starting material melted at 176–178°.

Androstane-11β-ol-3,17-dione (III).—The m.p. of the crystals obtained in fractions 10 to 12 suggested the presence of a mixture. Careful rechromatography of these fractions on a second silica gel column was carried out. Early eluates (with benzene-ether 7:3) yielded 36 mg. of a mixture melting at 167–197°. Of this fraction only 4 mg. (m.p. 212–217°) were not precipitable with digitonin, which indicated that the major portion consisted of a 3β-hydroxyl steroid, presumably compound II. Subsequent eluates of the same solvent mixture yielded 32 mg. of a solid, from which 20

mg. of a compound melting at 213–221° were obtained after crystallization from acetone-ether. Three recrystallizations raised the m.p. to 215–217/221–223°; a part of the crystals did not demonstrate the double m.p., but melted at the higher temperature only. The compound was then compared with the androstane-11β-ol-3,17-dione (m.p. 223–225°) obtained by Jeanloz *et al.* (1953) from an adrenal perfusion of Δ^4-androstene-3,17-dione. Mixed m.p. and the characteristic sulfuric acid reaction pointed to the identity of the substances. This was confirmed by the infrared spectra of the carbonyl regions.[6]

Androstane-3β,11β-diol-17-one (*IV*).—In fractions 13 to 17 material which did not melt up to 300° was found on crystallization with acetone-ether. No further investigation of the nature of this substance has been carried out. Small amounts of such high melting material have also been observed in other adrenal perfusions (Meyer, 1953a).

The crystals from fractions 18 to 24 were recrystallized from acetone-ether to yield 8 mg. platelets melting at 227–232°. The mother liquors were combined and rechromatographed. The ether eluates yielded after one crystallization 5 mg. of compound V with m.p. 184–190° (see next section). The material eluted with ether-ethyl acetate 19:1 melted after crystallization from acetone-ether (4 mg.) at 229–232°. By repeated crystallizations the m.p. of this compound was finally brought to a constant 232–235°. A sample of this substance treated at room temperature with concentrated sulfuric acid immediately developed an orange color. A sample of less pure material was converted into the acetate which melted at 227–229°. The melting points of the free compound and the acetate corresponded with those published for androstane-3β,11β-diol-17-one and its 3-monoacetate (v. Euw and Reichstein, 1942). Professor Reichstein then kindly made a comparison of compound IV with authentic material. No depression of the m.p. was seen upon admixture of both products. Upon receipt of a sample of the authentic material[7] the final identification by infrared spectrometry became possible. This sample demonstrated absorption bands in the solid state near 3422 (hydroxyls), 1722 (carbonyl at C17), 1040, 999, 978, and 906 cm^{-1} (some fingerprint bands). Our purest crystals of IV mixed with the authentic material in a ratio of approximately 1:1 gave the identical spectrum.[8]

Compound V.—The 5 mg. of compound V mentioned above and the 20 mg. of crystals obtained from fractions 25 to 30 of the first chromatogram

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[6] We are indebted to the late Dr. K. Dobriner for this analysis.

[7] We would like to thank Dr. T. Reichstein for his kind cooperation.

[8] These infrared determinations were made by Dr. H. Rosenkrantz on a Perkin-Elmer spectrometer (model 12C).

(see Table 1), both yielded needles melting at 190–192° when recrystallized from acetone-ether. The substances were only slightly soluble in chloroform and did not give a color when treated with 90% trichloracetic acid (Rosenheim, 1929) or conc. sulfuric acid. The identity of both fractions was established by infrared spectrometry. The spectrum contained a preponderance of vibrations of unsaturated H—C≡C-linkages.[8] Elemental microanalysis gave a C value of 61.17 and a H value of 5.59%. The molecular weight was determined (camphor) as 207.[9] From these data it was deduced that V was unlikely to be a steroid. Lack of adequate amounts prevented the further elucidation of the nature of this substance.

DISCUSSION

The principal reactions noted in the multicycle adrenal (*in vitro*) perfusions of the four 17-ketosteroids thus far performed will be briefly discussed. The stereochemically specific hydroxylation at the 11β-position

TABLE 2. MULTICYCLE ADRENAL PERFUSIONS OF 17-KETO-ANDROSTANE DERIVATIVES

Steroid	Structure at the 3- and 5-position	Per cent 11β-hydroxylated products isolated		Reference
		3—OH derivative	3=0 derivative	
Androstenedione	$\Delta^4-3=0$		26	Jeanloz *et al.*, 1953
Dehydroepiandrosterone	Δ^5-3β-OH	?	11	Meyer *et al.*, 1953
Androsterone	5α-3α-OH	+		Hechter *et al.*, 1950
Epiandrosterone	5α-3β-OH	1	3	Present paper

was found with each of these steroids. The extent of this reaction with these substrates occurred, however, in varying degree (Table 2). In the dehydroepiandrosterone perfusion only the 11-hydroxylated derivative of androstenedione, but not that of the starting material was isolated. The formation of the latter may have been minimized by the presence of an active oxidative side reaction (see below). The unsaturated conjugated configuration (the Δ^4-3-keto structure) yielded the largest amounts of the 11-hydroxylated product. Of the saturated derivatives, the 3-keto compound appeared to be more readily transformed than the 3-hydroxy compound. This was concluded from the epiandrosterone perfusion described here, where androstanedione (the 3-keto compound) was isolated in approximately one-third the amount of the starting material while the ratio of the concentrations of their 11-hydroxylated derivatives were in the reverse order. A similar trend in reactivity has been found with the C_{21}-steroids

[9] These analytical data were determined by Drs. G. W. Weiler and F. B. Strauss, Oxford, England.

(cf. Meyer, 1953b), and has been confirmed with the 11β-hydroxylase system of adrenal residue preparations by Hayano and Dorfman (1953).

The existence of a strong mechanism for the dehydrogenation of the hydroxyl group of dehydroepiandrosterone was noted in a previous perfusion. Such an oxidation of steroids with the Δ^5-3β-hydroxyl configuration has been investigated by Samuels *et al.* (1951) in various endocrine tissue slices of different species. The isolation of androstanedione in the present epiandrosterone perfusion represents an extension of the finding of a dehydrogenating system in adrenals to the saturated steroid nucleus. Recent experiments with the four isomeric 3-hydroxy-17-ketones and dehydroepiandrosterone revealed that the adrenal homogenate showed a distinct preferential dehydrogenation of the 3β-hydroxyl compounds irrespective of the linkage of the A/B nuclei; the 3α-hydroxyl structures were only minimally oxidized.[10] The reverse reaction, a hydrogenation of the 3-keto group to the 3α- or the 3β-hydroxy-5α-configuration has been observed in perfusions of appropriate derivatives in the corticosteroid series (Meyer, 1953a, b).

Other reducing mechanisms appear to be present in the adrenal-blood perfusion system. A stereospecific hydrogenation of the double bond of Δ^4-androstene-3,17-dione to the 5α-configuration has been found. The same reaction has been observed with other substrates (Meyer, 1953a). That blood is also capable of reducing the unsaturated ketonic grouping has recently been demonstrated (Meyer, 1953a).

SUMMARY

After a multicycle perfusion of epiandrosterone (androstane-3β-ol-17-one) through surviving isolated cow adrenal glands, three transformation products were identified. In order of decreasing yields these were: androstane-3,17-dione, androstane-11β-ol-3,17-dione, androstane-3β,11β-diol-17-one. The major reactions occurring in the course of the adrenal perfusions of 17-ketosteroids are discussed.

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THE INFLUENCE OF SOMATOTROPIN AND CORTICOTROPIN ON THE ISLETS OF LANGERHANS OF THE RAT[1]

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THE manner in which the anterior hypophysis affects the islets of Langerhans in the rat is not clear. An increase in the amount of islet tissue has been reported to occur after the administration of a saline extract of the anterior hypophysis (Richardson and Young, 1937) and after hypophysectomy (Krichesky, 1936). Except for a preliminary observation that treatment with corticotropin may induce histological evidence of beta cell stimulation (Baker, 1952), no attention has been given to the cytological effects of individual pituitary hormones.

The purpose of this report is (a) to describe the cellular changes induced in the islets by somatotropic and corticotropic preparations administered singly and in combination, and (b) to relate these effects to the presence or absence of glycosuria and to alterations in body weight and in the excretion of non-protein nitrogen. Clarification of the effects induced by somatotropin on the islets is particularly important because Young (1951) has postulated that an increased secretion of insulin is necessary for the hormone to stimulate growth while Salter and Best (1953) induced growth of hypophysectomized rats by the injection of insulin. Thus, it is possible that the islets of Langerhans mediate in part the growth-promoting action of somatotropin. It was hoped that correlation of beta cell cytology with urinary excretion of glucose would indicate the relative importance of somatotropin, corticotropin and the blood sugar level in regulating the secretion of insulin by the beta cells.

MATERIALS AND METHODS

Preparations with somatotropic properties were made from ox pituitary glands by a procedure which involves salt fractionation and isoelectric precipitation (Li, Evans and

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[2] Summer Research Fellow of the University of Michigan Medical School.

Simpson, 1945). Injection of a total dose of 5 mg. into hypophysectomized female rats revealed no histological evidence of corticotropic, thyrotropic or gonadotropic activity. Also, the absence of lactogen was indicated by the failure of 10 mg. to stimulate the crop sac of one month-old squabs. Corticotropic preparations were made from sheep hypophyses by a method which includes acid-acetone extraction, fractionation with NaCl and heating in an acid medium (Li, Evans and Simpson, 1943; Li, Liddle, Reinhardt and Bennett, 1951). When 36 mg. of the final preparation were administered to immature hypophysectomized male rats in divided doses, no evidence of thyrotropic or gonadotropic activity was obtained as determined by weight of the end-organs. These preparations were treated with 0.2 N HCl and heat in order to reduce antidiuretic and pressor activity. Under test conditions the effects exerted on blood pressure and excretion of water were comparable with those of physiological saline (Reinhardt and Li, 1951).

Adult female Sprague-Dawley rats, which weighed 225 to 325 gm. at the start of the experiment, were used for the study of the effects of somatotropin and corticotropin on the islets of Langerhans. They were fed a medium carbohydrate diet (Bole, Baker, Ingle and Li, 1951) by stomach tube, according to the method of Reinecke, Ball and Samuels (1939). The animals were adapted to the injection apparatus and tube-feeding during a 7-day control period after which the hormones were administered by continuous subcutaneous injection for 10 days. During the control and experimental periods each animal was weighed daily, and the NPN and glucose content of 24-hour urine samples were determined. These observations will be the subject of a subsequent report.

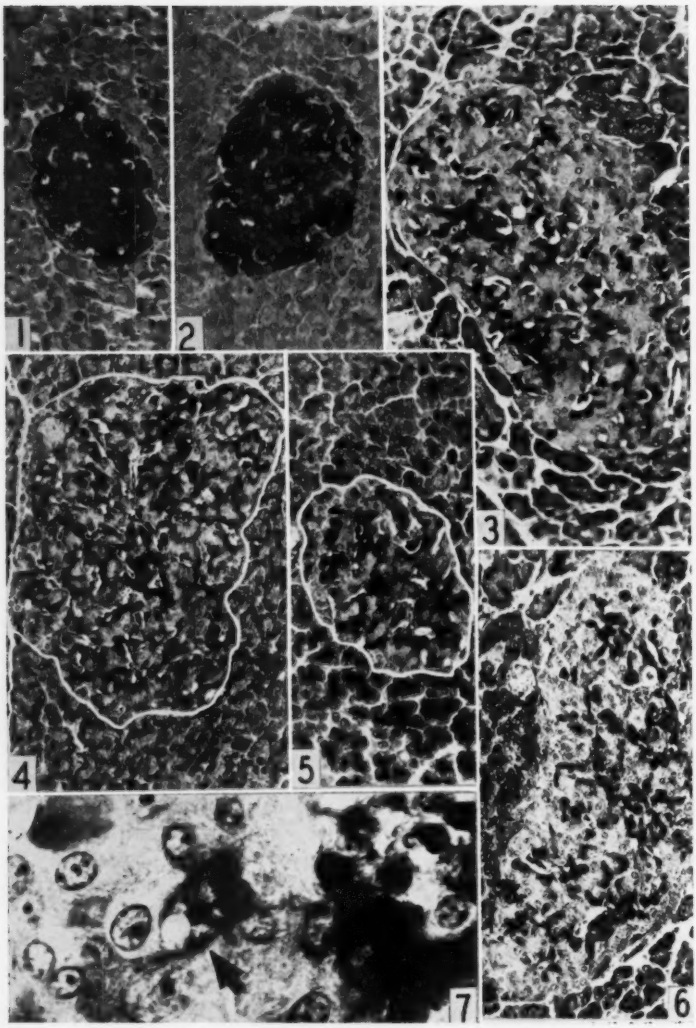
Fifty-four animals were used in 9 separate experiments. Of the 6 animals in each experiment, one received no injections, one received 4 cc. of 5% gelatin or physiological saline daily; one, somatotropin; one, corticotropin; and 2 received both hormones. In 4 experiments, the daily doses of somatotropin and corticotropin were 1 mg. and 0.5 mg., respectively; in two experiments, 1 mg. of each hormone was given; and in three experiments, 2 mg. and 0.5 mg. At autopsy, the pancreas was fixed in Bouin's fluid and after sectioning at 5μ, stained with Gomori's (1950) aldehyde-fuchsin and counterstained with the Masson procedure. The beta cell granules appeared a deep purple and those of the alpha cells a light red. The extent of degranulation in beta cells was estimated as 0 to ++++, the latter indicating almost total absence of granulated beta cells from the islets. The size of alpha cells was determined in 5 experiments by measuring the length and width of 100 alpha cells in each pancreas under oil immersion with an ocular micrometer and letting the product of the two measurements represent the area of the cell. The mean of these areas for each animal was used in the statistical treatment.

OBSERVATIONS

Beta Cells

Controls. Most of the beta cells of control rats contained purple granules which stained intensely with aldehyde fuchsin. There was some variation in the number of granules found in individual cells but generally the granules were densely arranged (Fig. 1). Completely degranulated cells were exceedingly rare. The beta cells of the control animals which received gelatin did not differ from those of the uninjected controls. Mitotic figures were seen rarely. None of the control rats exhibited glycosuria.

Somatotropin. The effect of somatotropin on beta cells was variable and probably insignificant. In 5 experiments no histological alteration of the



EXPLANATION OF FIGURES

All tissues were stained with aldehyde-fuchsin and the Masson procedure. The islets in figures 4 and 5 are outlined with ink. Figures 1–4 are from one experiment, Figures 5 and 6 from another. Figures 1–6, ×200.

FIG. 1. Islet from a control rat, 4 cc. of 5% gelatin daily.

FIG. 2. Islet from a rat which received 1 mg. of somatotropin daily.

FIG. 3. Islet from a rat which received 0.5 mg. of corticotropin daily, showing extensive

(*continued on facing page*)

beta cells occurred. In 4 experiments varying degrees of degranulation and hypertrophy of beta cells were present (rated + to ++) (Fig. 2). The presence or absence of effect bore no relationship to dosage. In only 2 of these 9 animals was hyperplasia indicated by the presence of increased numbers of mitotic figures.

These differences in response of the islets were not related to variations observed in urinary NPN or in rate of increase in body weight. Beta cells were mildly hypertrophied and degranulated in one animal which exhibited marked growth (33 gm. in 10 days) and reduction of urinary nitrogen, but were unchanged in 2 others which gained 27 and 29 gm. Two animals with some hypertrophy and hyperplasia of beta cells gained only 13 gm. and did not show a reduced nitrogen excretion. Glycosuria was not observed.

Corticotropin. Corticotropin caused an enlargement of the islets accompanied by hypertrophy and degranulation of beta cells (Fig. 3), the treatment being ineffective in only one experiment. The magnitude of the change was variable, being rated + in 4 rats; ++ in one; +++ in 3; and ++++ in one. In the rats with greatest modification of the beta cells, the nucleoli and negative image of the Golgi apparatus were enlarged.

An increase in number of beta cells also contributed to islet hypertrophy, there being several apparent sources for the new cells. Beta cells multiplied by cellular proliferation, mitotic figures being observed frequently. Beta cells also appeared to arise by direct transformation from acinar cells as previously described (Baker, 1952). In addition, it is probable that beta cells arose by transformation of ductular epithelium since purple granules were found in some cells of the latter and small islets were occasionally continuous with the epithelium of ducts.

Other abnormal changes appeared in the beta cells. Occasionally the nucleus was enlarged and contained a vacuole which in its extreme development filled most of the interior (Fig. 3). The nucleolus was enlarged and the nuclear membrane thickened. This effect was limited to a small percentage of the hypertrophied degranulated beta cells. In one rat, showing +++

degranulation and enlargement of beta cells. Some nuclei are enlarged and vacuolated (arrows). A mitotic figure is at the lower arrow.

FIG. 4. Islet (outlined by white line) from a rat which received 1 mg. of somatotropin and 0.5 mg. of corticotropin daily. The beta cells are changed less than in Figure 3.

FIG. 5. Islet (outlined by white line) from a rat which received 0.5 mg. of corticotropin daily.

FIG. 6. Islet from an animal which received 1 mg. of somatotropin and 0.5 mg. of corticotropin daily, showing intensification of beta cell degranulation and enlargement as compared with Figure 5.

FIG. 7. Granulated beta cell which shows marked cytoplasmic vacuolation (arrow). From the same animal as Figure 6. ×1330.

beta cell hypertrophy and degranulation, most of the few beta cells which retained their granules exhibited extreme cytoplasmic vacuolation (Fig. 7). The nucleus was either normal or exhibited a loss of affinity for the stain, in certain instances having disappeared entirely.

Glycosuria occurred in 5 rats but was usually transitory and existed at the time of sacrifice in only 2 animals. In the latter 2 cases, beta cell change was rated +++ and ++++. Generally, the most significant hypertrophy and degranulation of beta cells occurred in those animals which excreted glucose at or near the time of sacrifice. One rat which was glycosuric only on the third and seventh days of injection exhibited unchanged beta cells at autopsy. The magnitude of beta cell alteration was most pronounced in those animals showing the greatest weight loss and rise in nitrogen excretion.

Somatotropin and corticotropin. The effect exercised on the islets by both hormones administered simultaneously was qualitatively the same as that produced by corticotropin alone. Two rats showed no change from the controls. Of the remainder, beta cell degranulation and hypertrophy was rated + in 9, ++ in 4, +++ in one, and ++++ in one. One rat exhibited cytoplasmic vacuolation of granulated beta cells. Glycosuria occurred in 7 of these rats, while in 5 it was transitory and not present at autopsy. The two which were glycosuric at sacrifice are the ones which showed +++ and ++++ beta cell hypertrophy and degranulation.

These experiments do not reveal whether concurrent administration of somatotropin augments or depresses the islet changes induced by corticotropin. In 4 experiments, combined treatment with both hormones increased the extent of beta cell hypertrophy and degranulation as compared with that induced by corticotropin alone (Figs. 5, 6); in 4 experiments somatotropin appeared to suppress the effects induced by corticotropin (compare Figs. 3 and 4). The remaining animals treated with both hormones did not differ significantly from those injected with corticotropin alone. When somatotropin alone produced a considerable increase in body weight (27–36 gm.), it augmented the effect of corticotropin upon the beta cells; likewise, it appeared to be inhibitory if less effective in increasing body weight (3–14 gm.).

Alpha Cells

The size of alpha cells was increased by treatment with either hormone alone, or both together, the difference between the means of the treated and control animals being statistically significant only in the case of somatotropin (Table 1).

The mean areas of the alpha cells for all animals were grouped according to the magnitude of stimulation of the beta cells, i.e., 0 to ++++, and

TABLE 1. THE EFFECT OF HORMONE TREATMENT ON SIZE OF ALPHA CELLS

	Controls	Somatotropin	Corticotropin	Som. and cort.
Number of rats	9	5	5	9
Mean area (μ^2) of alpha cells	53.1 ± 0.5[1]	57.2 ± 1.2	58.6 ± 2.0	56.9 ± 1.6
P value	—	< .05[2]	> .05	> .05

[1] Standard error of the mean = $\sqrt{\frac{\Sigma d^2}{N(N-1)}}$

[2] Difference between the mean and that of the controls. Behrens-Fisher test.

the means of the mean area of each animal in the groups rated + to ++++ were compared with the mean of the rats rated 0. It is probable that some increase in size of alpha cells accompanied beta cell stimulation as revealed by cytological study (Table 2) although the increase is not statistically significant in all groups. The alpha cells in the islets with greatest change in the beta cells seemed to be more granular and to constitute a greater proportion of the parenchyma. The enlargement of alpha cells induced by somatotropin (Table 1) is an exception since this hormone had little effect on beta cells.

DISCUSSION

The considerable variation in effects elicited by somatotropin and corticotropin in different experiments demonstrates that we have been unsuccessful in controlling all interfering factors. It is probable that variations in the responsiveness of different rats and in potency and purity of the hormone preparations were of particular importance.

Nevertheless, several points regarding the functional interrelations of the anterior hypophysis and the islets of Langerhans of the rat may be drawn from these observations. First, no supporting evidence was obtained for the hypothesis that somatotropin exerts a specific stimulatory action on the beta cells and promotes growth through the accelerated se-

TABLE 2. THE RELATION OF ALPHA CELL SIZE TO BETA CELL HYPERTROPHY AND DEGRANULATION

	Rating of beta cell change				
	0	+	++	+++	++++
Number of rats	13	6	4	3	2
Mean area (μ^2) of alpha cells	53.4 ± 0.4[1]	57.8 ± 1.7	56.7 ± 2.6	61.2 ± 2.1	59.9 ± 1.4
P value	—	< .05[2]	> .05	< .05	> .05

[1] Standard error of the mean = $\sqrt{\frac{\Sigma d^2}{N(N-1)}}$

[2] Difference between the mean and that of the group rated 0. Behrens-Fisher test.

cretion of insulin. Cytological modification of beta cells by somatotropin was slight and inconstant. Increases in body weight and nitrogen retention occurred in the absence of beta cell stimulation. Other evidence is also incompatible with the hypothesis that somatotropin stimulates the beta cells. Anderson and Long (1947) found that perfusion of isolated rat pancreas with somatotropin inhibited the secretion of insulin which occurs in response to a high level of blood glucose. The observation of Marks and Young (1940) that pituitary extract increases the content of insulin in the rat pancreas may indicate an inhibited release of the hormone rather than stimulation.

In the apparent resistance of its beta cells to stimulation by somatotropin, the rat differs from the dog and cat. Crude extracts of the anterior hypophysis (Young, 1938) and purified somatotropin (Houssay and Anderson, 1949; Cotes, Reed and Young, 1949; Campbell, Davidson and Lei, 1950) induce temporary or permanent diabetes in the latter species. If permanent diabetes occurs, the beta cells are hydropic or destroyed following a period of excessive secretory activity (Ham and Haist, 1941).

Second, with somatotropin being regarded by some investigators as a stimulator of beta cells, it should be noted that in the rat, corticotropin is far more effective. In our experiments, 0.5 mg. of corticotropin produced greater modification of beta cells than did 2.0 mg. of somatotropin. This observation can be correlated with the action of these two pituitary factors on carbohydrate metabolism. Rats are rather resistant to the diabetogenic action of somatotropic preparations (Young, 1951) but may exhibit hyperglycemia and glycosuria if concurrently given a high carbohydrate diet and an intake of corticotropin which by itself is too low to induce these effects (Engel, Viau, Coggins and Lynn, 1952). In contrast, hyperglycemia and glycosuria may appear in rats treated with adrenal steroids (Ingle, Sheppard, Evans and Kuizenga, 1949) or corticotropin (Ingle, Li and Evans, 1946). Likewise, corticotropin is more effective than somatotropin in enhancing the excretion of glucose in rats made diabetic with alloxan (Bennett and Li, 1947).

Third, cytological evidence of extreme beta cell stimulation in the rats treated with corticotropin, or corticotropin and somatotropin, was correlated with the presence of glycosuria at or near the time of autopsy. This suggests that the level of blood sugar (or insulin) rather than a pituitary secretion regulates the secretory activity of beta cells. The histology of these islets was strikingly similar to that observed by Friedman and Marble (1941) in the pancreatic remnant of rats previously subjected to partial pancreatectomy, and by Wissler, Findley and Frazier (1949) in rats force-fed a high carbohydrate diet. Haist *et al.*, (1949) induced hyperplasia of the islets in hypophysectomized rats by administration of glu-

cose. In guinea pigs injected continuously with glucose, Woerner (1938) described similar cytological changes in beta cells and the same 3 sources for the development of new beta cells. In certain of our rats whose transitory glycosuria had disappeared at the time of autopsy, the beta cells were unchanged. A rapid return to a normal state of activity and cytological appearance would be expected, since Peterson (1949) demonstrated that beta cells require only 48 hours for complete regranulation following degranulation resulting from injected glucose.

Our preliminary observations of changes in alpha cell size do not permit an evaluation of the manner in which they respond to pituitary activity.

SUMMARY

A somatotropic preparation which increased body weight and nitrogen retention in adult female rats force-fed a moderate carbohydrate diet, did not affect significantly the size of the islets of Langerhans nor the cytology of the beta cells.

A corticotropic preparation increased the number and size of the islets and induced enlargement and degranulation of the beta cells. A transitory glycosuria occurred in some animals and when present at autopsy, was accompanied by cytological evidence of the most intense beta cell stimulation.

Concurrent administration of somatotropic and corticotropic preparations induced variable effects on the islets. Some increase in size of the alpha cells was brought about by both preparations.

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EFFECT OF STEROIDS ON OXIDATION OF PROLINE BY KIDNEY OF ADRENALECTOMIZED AND HYPOPHYSECTOMIZED RATS

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OF THE enzymatic reactions which undergo modification in the tissues of animals following adrenalectomy, one of the most sensitive is the proline oxidase system of rat kidney. Umbreit and Tonhazy (1951) have shown that the ability of this tissue to oxidize proline declines significantly by the third day after operation and reaches a minimal value in about one week. Administration of cortisone (Kendall's compound E; 4-pregnene-17α,21-diol-3,11,20-trione) to the animal serves to maintain this function, or to return it to normal if it has been allowed to decrease by withholding hormonal treatment following adrenalectomy. These authors concluded from their data that cortisone serves in the formation rather than the activation of the enzyme.

Our main aim in pursuing the investigation described here was to determine whether a relation exists between steroid structure and activation of the proline oxidase system. For this purpose we have used four cortical steroids whose physiological and pharmacological properties are well known, as well as the 11-epimer of Kendall's compound F (epi-F; 4-pregnene-11α-17α,21-triol-3,20-dione), which has received to date little notice in the biological literature. Since hypophysectomy is followed by adrenal cortical atrophy and, consequently, by a decrease in endogenous production of steroids, we were also interested in learning whether the proline oxidase system of rat kidney is affected in hypophysectomized rats in the same way as in adrenalectomized animals.

EXPERIMENTAL

Adult male albino rats weighing about 200 gm. were bilaterally adrenalectomized for us by Mr. T. Bielinski. Hypophysectomized male albino rats were purchased from Hormone Assay Laboratories, Chicago. The animals were maintained on a complete diet, the adrenalectomized rats receiving 1% NaCl solution to drink instead of water.

Three main experimental series were studied, according to the following

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regimes: I. ("Maintenance" schedule) Adrenalectomized rats; injections begun 1–2 days post-operatively and continued for 8 days (4 replicates). II. ("Recovery" schedule) Adrenalectomized rats; injections begun 3 weeks post-operatively and continued for 8 days (6 replicates). III. ("Recovery" schedule) Hypophysectomized rats; injections begun 2 weeks after operation and continued for 8 days.

The steroids (used as acetates) were injected subcutaneously in daily doses per animal of 1 mg. dissolved or suspended in 0.4 ml. propylene glycol. Control animals received injections of 0.4 ml. of the solvent. In all three series the animals were sacrificed by stunning about 4 hours after the last injection. The whole kidney was homogenized in 9 volumes of cold, isotonic KCl and added to Warburg flasks. The final concentrations of components of the reaction mixture were: K phosphate buffer, pH 7.4, 0.007 M; KCl, 0.115 M; K adenylate, pH 7.4, 0.001 M; $MgCl_2$, 0.0033 M; L-proline, neutralized to pH 7.4, 0.0166 M; 60 mg. of tissue (wet weight) per flask. Final volume was 3.0 ml. The center-well contained KOH solution, to absorb any evolved CO_2. The values for oxygen uptake were routinely corrected for "endogenous" gas consumption determined in flasks similarly prepared except for omission of substrate. Thus, tabulated data represent "extra oxygen consumption" (due to presence of proline) during the first 30 minutes following equilibration of the flasks at 37°. The gas phase was air.

RESULTS

The data obtained in experiments with adrenalectomized rats are shown in Table 1. Part A gives the means of observations made with rats receiving the various treatments and part B provides the statistical analysis (Snedecor, 1946) of these data, including a statement of the standard error of differences between means. Of the steroids administered on a "maintenance" schedule, cortisone, hydrocortisone (Kendall's compound F; 4-pregnene-11β, 17α,21-triol-3,20-dione), and 11-desoxycorticosterone (4-pregnene-21-ol-3,20-dione) preserved the proline oxidizing ability at levels comparable to that of the sham-operated controls. Reichstein's substance S (11-desoxy-17-hydroxycorticosterone; 4-pregnene-17α,21-diol-3,20-dione) maintained the enzyme system at levels intermediate between sham operated and adrenalectomized controls, but significantly lower than the former. On the recovery schedule, homogenates of kidneys obtained from rats receiving cortisone and hydrocortisone, respectively, showed activity comparable to those obtained from the sham-operated controls. Homogenates of kidneys of animals receiving Reichstein's substance S were about as active as those of the adrenalectomized controls. The effect of 11-

TABLE 1. EFFECT OF STEROIDS ADMINISTERED *in vivo* ON PROLINE OXIDIZING ABILITY OF ISOTONIC KIDNEY HOMOGENATES

A. Table of means of extra O_2 uptake, due to presence of substrate

Treatment	Steroid injected	Schedule			
		Maintenance		Recovery	
		No. of rats	Extra O_2 µl./30 min.	No. of rats	Extra O_2 µl./30 min.
Sham-operated	—	4	74	6	70
Adrenalectomized	—	4	33	6	50
Adrenalectomized	Cortisone	3	72	6	72
Adrenalectomized	11-desoxycorticosterone	4	62	4	32
Adrenalectomized	Hydrocortisone	4	59	6	64
Adrenalectomized	Reichstein's substance S	3	53	5	53

B. Analysis of variance of data in Table 1A

Source of variation	Maintenance			Recovery		
	Degrees of freedom	Mean square	F	Degrees of freedom	Mean square	F
Replicates	3	3767	21.9*	5	1509	8.1*
Treatments	5	1044	6.1*	5	1364	7.3*
Error	13	172	1	22	187	1
Standard Error:						
Mean of 4		6.6 µl.			—	
Mean of 6		—			5.6 µl.	
S.E. of a difference between:						
2 means of 4		9.3			—	
2 means of 6		—			7.9	

* Highly significant source of variation, $P < 0.01$

desoxycorticosterone was to depress markedly the proline-oxidizing ability of the kidney, even beyond the level observed in these controls.[2]

The effect of 11-desoxycorticosterone in rats on the "recovery" schedule was thus in marked contrast with that seen in the "maintenance" experiment, whereas the other steroids possessed qualitatively similar actions in both tests. The possibility was considered that 11-desoxycorticosterone displays activity in the "maintenance" type of experiment through some nonspecific mechanism, such as its ability to improve the general condition of the adrenalectomized rat and to favor its growth and retention of nitrogen (cf. Ingle, 1950). Since the relation of the proline oxidase system of kidney to the nutritional status of the animal has not been previously studied,

[2] Corticosterone acetate was tested in one adrenalectomized rat. Oxygen uptake by its homogenized kidney was 66 µl./30 min. Kidney tissue from a paired sham-operated control was 72 µl./30 min. The steroid was kindly supplied to us by Dr. W. Haines, Upjohn Co., Kalamazoo, Mich.

we conducted an experiment with young rats (initial mean weight = 103 gm.) at two widely different levels of nutrition. The animals were fed on a minimal amount of stock diet (not more than 3.0 gm. per day) for one week, by which time they had lost 29% of their initial body weight. Two groups of four rats were then sacrificed and the oxidation of proline by homogenates of their kidneys was measured. Two other groups of animals were then returned to full feeding for a second week at the end of which they were sacrificed (final mean body weight = 120 gm.).

The kidney of repleted animals oxidized proline at almost twice the rate observed with kidney of the starved, or nitrogen-depleted, group (Table 2). This result parallels the effect seen with 11-desoxycorticosterone on

TABLE 2. EFFECTS OF STARVATION AND RE-ALIMENTATION ON PROLINE-OXIDIZING ABILITY OF KIDNEY OF NORMAL RATS

Group	Treatment	Mean O_2 uptake μl./30 min.	
A	Starved 1 week	72	65
B	Same as A	61	
C	Starved first week, re-fed second week	106	119
D	Same as C	133	

Source of variation	D.F.	Mean square	F
Total	15		
Starved vs. Re-alimented	1	10,972.0	33.2*
Replicates	2	979.0	2.9
Error	12	330.0	1

* Very highly significant, $P < 0.001$

the "maintenance" schedule and may be a sufficient explanation of it. On the other hand, it is probable that the effect of cortisone and hydrocortisone on proline oxidizing ability is mediated through some qualitatively different mechanism since these two steroids tend to drive the adrenalectomized rat into a state of negative nitrogen balance.

A separate series of animals, treated with epi-F was studied on the "recovery" schedule. The volumes of O_2 taken up by kidney of (a) adrenalectomized control rats, and (b) adrenalectomized rats treated with epi-F were (mean $\pm$ standard error): (a) 60 ± 18 μl. (5 rats), and (b) 37 ± 6 μl. (6 rats). The difference in rates is not significant at the 5% level. The reason for the unusually large variation in the control group is not apparent.

As for hypophysectomized rats (Table 3) the proline-oxidizing ability of their kidney was low compared to the rates usually seen in intact animals (cf. Table 1, "sham-operated"). Of the three steroids tested only cortisone was capable of bringing about a substantial and significant increase.

DISCUSSION

Taking the data as a whole one may conclude that the level of the proline-oxidase system in rat kidney is ordinarily subject to regulation by at least two mechanisms: a nutritional (nitrogen balance?) and a pituitary-adrenal relationship. Kidney of animals lacking either the hypophysis or the adrenal gland exhibits a decreased ability to oxidize proline, as compared to normal animals. This ability can be restored to normal by appropriate treatment of the animal with cortisone or hydrocortisone, but not by 11-desoxycorticosterone, Reichstein's substance S or epi-F. Adrenalectomized rats maintained on 11-desoxycorticosterone have, it is true, a

TABLE 3. EFFECT OF STEROIDS ADMINISTERED *in vivo* ON PROLINE OXIDIZING ABILITY OF ISOTONIC HOMOGENATES OF KIDNEY OF HYPOPHYSECTOMIZED RATS

Steroid injected	No. of rats	Mean O_2 uptake μl./30 min.	Standard error of the mean
None	10	39	5.3
Cortisone	9	83	9.8
11-Desoxycorticosterone	7	46	8.5
Reichstein's substance S	6	32	5.4

Significant differences (P less than 0.05): Cortisone greater than hypophysectomized control, 11-desoxycorticosterone and Reichstein's substance S, respectively.

normal level of the proline oxidase system in their kidney but this can be related to the improvement in nutritional state of such animals. The difference in the respective actions of 11-desoxycorticosterone and cortisone is pointed up in the experiments on the recovery schedule with adrenalectomized (Table 1) and hypophysectomized rats (Table 3). In both of these, injection of cortisone brought about a return of the proline oxidase system to normal levels, whereas 11-desoxycorticosterone caused a further decline in the level of the system in the adrenalectomized rats and had no effect at all in the hypophysectomized animals.

It should be emphasized again that the effects of cortisone and hydrocortisone are observed only when they are administered to the whole animal. Umbreit and Tonhazy (1951) attempted unsuccessfully to activate the proline oxidase system using additions of cortisone and its acetate *in vitro*. Since then a water-soluble form of cortisone has become available, *viz.* the tricarballylic acid ester. Even with the advantage of enhanced solubility of this form of cortisone we were also unable to demonstrate any *in vitro* activation.

SUMMARY

1. The oxidation of proline has been studied in isotonic homogenates of kidneys of adrenalectomized and of hypophysectomized rats. The effect

of prior treatment of the animals with various cortical steroids has been determined.

2. In adrenalectomized rats injections of cortisone or hydrocortisone permitted retention of proline-oxidizing ability of kidney at normal levels. Under certain conditions 11-desoxycorticosterone did the same but its action could be related to indirect, non-specific effects. Reichstein's substance S and compound epi-F were without activity.

3. Hypophysectomized animals displayed a decreased ability of their kidney to oxidize proline. This could be returned to normal levels by treatment of the animal with cortisone, but not with 11-desoxycorticosterone or Reichstein's substance S.

4. Kidney of intact rats which were first starved and then full-fed had a significantly higher level of the proline oxidase system than kidney of starved rats.

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THE *IN VIVO* METABOLISM OF 21-DESOXYCORTISONE[1]

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PREVIOUS studies of urinary metabolites in man have shown that progesterone (Ungar *et al.*, 1951, Kyle and Marrian, 1951), desoxycorticosterone (Cuyler *et al.*, 1940), 11-dehydrocorticosterone (Mason 1948), hydrocortisone (Burstein, *et al.* 1953a) and cortisone (Burstein, *et al.* 1953b) (Dobriner 1951) predominantly are reduced to the ring A saturated C-21 derivatives possessing the pregnane (5β) configuration. The C-19 17-ketosteroid metabolites arising from hydrocortisone and cortisone (Dobriner, 1951) have likewise been shown to be mainly of the etiocholane (5β) series.

The purpose of the present paper is to report on the study of the C-21 and C-19 urinary steroid metabolites arising from the administration of 21-desoxycortisone (Δ^4-pregnen-17α-ol-3,11,20-trione).[2] The results of the present study also demonstrate the conversion of this compound to C-21 and C-19 saturated derivatives of the 5β configuration.

METHODS

Two arthritic women were treated with 1 and 3 gm. 21-desoxycortisone,[3] respectively. Urine, which constituted the control specimens, was collected for three days prior to the steroid treatment. Both control and experimental urines were subjected to hydrolysis, extraction and separation into ketonic and non-ketonic material in the manner previously described. The ketonic material obtained by β-glucuronidase hydrolysis and continuous extraction with methylene chloride at pH 1 for 24 hours is termed "cold hydrolysis" fraction. The ketonic material resulting from the hot acid hydrolysis is

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[2] 21-Desoxycortisone was generously supplied by Dr. Albert Raymond of the Searle Company, Inc., Skokie, Illinois.

[3] The clinical aspects of this study were supervised by Dr. Harry Freeman of the Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

termed the "hot hydrolysis" fraction. The non-ketonic fractions were not studied but were reserved for future investigation. The crude ketonic extracts were subjected to paper chromatography using propylene glycol (P.-G.) impregnated filter paper. Toluene (T.) and ligroin (L.) were employed as mobile phases. The steroid zones detected by the Zimmermann and dinitrophenyl hydrazine reagents were cut out and eluted in the manner previously described.[1]

Identification of the steroids was achieved by their mobility on paper, melting points and by infra-red spectrometry. (We are indebted to Dr. H. Rosenkrantz for the valuable interpretation of the infra-red absorption spectra.) All melting points reported were determined with the Kofler Micro Hot Stage melting point apparatus and are uncorrected.

EXPERIMENTAL AND RESULTS

Patient No. 1

A 41 year old woman with rheumatoid arthritis was given 3 gm. of 21-desoxycortisone orally during a period of 20 days: 200 mg. daily for the first 10 days and 100 mg. thereafter. Isolation of steroid metabolites was performed on a urine sample which represented 2.35 gm. of administered 21-desoxycortisone. The isolation studies are summarized in Tables 1–3.

Control Urine.—The "cold hydrolysis" fraction was chromatographed on paper in the L.-P.G. system (24 hours). Two zones were visualized by the Zimmermann reagent (1-I and 1-II, Table 1). Zone 1-I had the mobility

TABLE 1. STEROIDS EXCRETED IN THE CONTROL PERIOD URINE OF PATIENT NO. 1

Chromatogram zone	Chromatography system	Time hours	Distance of zone from starting line cm.	17-KS value[1] mg.	Compound	Identifying Characteristics
			"Cold Hydrolysis" Fraction			
1-I	L.-P.G.[2]	24	1.0– 2.0	0.58	11β-Hydroxyandrosterone	Mobility[3]
1-II	L.-P.G.	24	12.0–16.5	0.50	Etiocholan-3α-ol-17-one	Mobility
			"Hot Hydrolysis" Fraction			
2-I	L.-P.G.	24	1.5– 4.5	0.10	Etiocholan-3α-ol-11,17-dione	Mobility
2-II	L.-P.G.	24	11.0–16.1	0.27	Etiocholan-3α-ol-17-one	Mobility[4]
2-III	L.-P.G.	24	20.0–27.3	0.26	$\Delta^{9(11)}$-Androsten-3α-ol-17-one	Mobility[5]

[1] 17-Ketosteroids as determined by the Zimmermann reaction.

[2] Ligroin-propylene glycol.

[3] Possessing the mobility of the respective authentic compound. The eluates of zones 1-I, 1-II and 2-I had mobilities identical with the respective authentic compounds in mixed chromatograms.

[4] Only 20% of this material reacted with perbenzoic acid (6) to yield a slower moving epoxide, indicating the possible maximum quantity of only 0.05 mg. of $\Delta^{9(11)}$-etiocholen-3α-ol-17-one.

[5] Ninety per cent of this fraction reacted with perbenzoic acid to yield the slower moving epoxide.

of 11β-hydroxyandrosterone and the eluate of this zone was chromatographically identical in a mixed chromatogram with the latter steroid. The eluate of zone 1-II in a mixed chromatogram possessed a mobility identical with that of etiocholan-3α-ol-17-one. No androsterone was detected.

The "hot hydrolysis" fraction was chromatographed in the L.-P.G. system (24 hours) and three zones were visualized by the Zimmermann reagent (2-I, 2-II, 2-III, Table 1). The eluate of zone 2-I had a mobility identical with that of etiocholan-3α-ol-11,17-dione in a mixed chromatogram. Zones 2-II and 2-III had the mobilities of etiocholan-3α-ol-17-one and $\Delta^{9(11)}$ androsten-3α-ol-17-one, respectively. Despite repeated chromatography on paper, these fractions were too impure for identification by infra-red spectrometry.

Androsterone and $\Delta^{9(11)}$-androsten-3α-ol-17-one do not separate in the L.-P.G. system. Treatment of material contained in zone 2-III with perbenzoic acid according to Lieberman *et al.* (1952) converted 90% of this material into a 17-ketosteroid which had a mobility on paper expected for the $\Delta^{9(11)}$-androsten-3α-ol-17-one epoxide. Only about 20% of the etiocholan-3α-ol-17-one (zone 2-II) found in the "hot hydrolysis" fraction reacted with perbenzoic acid to yield the slower moving epoxide, which indicated a possible maximum quantity of only 0.05 mg. of $\Delta^{9(11)}$-etiocholen-3α-ol-17-one.[4]

Experimental Urine.—"Cold Hydrolysis" Fraction.—The neutral ketonic extract was chromatographed in the T.P.-G. system (24 hours). A zone which gave a brown-purple coloration with the Zimmermann reagent was detected (3-I, Table 2). Following elution, 7.1 mg. of crude crystalline material was obtained. Three successive crystallizations from acetone yielded 2.6 mg. colorless crystals which melted at 194–197°. The melting point upon admixture of an authentic sample of pregnane-3α,17α-diol-11,20-dione (m.p. 200–201°) was 198–199°. The infra-red spectrum of the crystals and the mother liquors was identical with that of an authentic sample of pregnane-3α,17α-diol-11,20-dione, generously supplied by Dr. T. F. Gallagher of the Sloan-Kettering Institute, New York.

A zone which was located 7.3–9.5 cm. from the starting line gave an orange dinitrophenylhydrazone on paper. Several ill-defined zones nearer to the starting line, giving a bluish color with the Zimmermann reagent on paper, were detected. These zones contained too little material for identification.

The material which migrated faster than zone 3-I was rechromato-

[4] We are indebted to Dr. Betty Rubin for carrying out the perbenzoic acid reactions and the Zimmermann color determinations.

TABLE 2. STEROIDS ISOLATED FROM THE URINE OF PATIENT NO. 1 FOLLOWING THE ADMINISTRATION OF 2.35 GM. 21-DESOXYCORTISONE

Chromatogram zone	Chromatography system	Time hours	Distance of zone from starting line cm.	Weight of material isolated mg.		Compound	Identifying Characteristics
				Crude	Crystals		
"Cold Hydrolysis" Fraction							
3-I	T.-P.G.[1]	24	10.5–15.5	7.1	2.6	Pregnane-3α,17α-diol-11,20-dione	M.P.194–197° Infra-red spectrum
4-I	L.-P.G.[2]	72	1.0– 3.0	3.4	1.0	Etiocholane-3α, 11β-diol-17-one	M.P.200–228° Infra-red spectrum
4-II	L.-P.G.	72	6.1– 7.4	0.3		11β-Hydroxyandrosterone	Mobility[3]
4-III	L.-P.G.	72	8.0–10.0	1.0		Etiocholan-3α-ol-11,17-dione	Mobility Infra-red spectrum
4-IV	L.-P.G.	72	12.0–14.0	0.6		11-Ketoandrosterone	Mobility
5-I	L.-P.G.	24	8.4–12.0	3.5		Etiocholan-3α-ol-17-one	Mobility Infra-red spectrum
"Hot Hydrolysis" Fraction							
6-I	L.-P.G.	24	2.8– 4.2	3.9		Etiocholan-3α-ol-11,17-dione	Mobility Infra-red spectrum
6-II	L.-P.G.	24	5.5– 7.1	5.7		?	Giving a brownish-purple Zimmermann reaction.
6-III	L.-P.G.	24	10.2–12.0	2.3		?	Giving a brownish-purple Zimmermann reaction
6-IV	L.-P.G.	24	18.5–21.5	4.2		$\Delta^{9(11)}$-Etiocholen-3α-ol-17-one	Mobility Infra-red spectrum
6-V	L.-P.G.	24	29.5–33.0	2.8		$\Delta^{9(11)}$-Androsterone-3α-ol-17-one	Mobility Infra-red spectrum

[1] Toluene-propylene glycol.
[2] Ligroin-propylene glycol.
[3] Possessing the mobility of the respective authentic compound. Fraction 4-II was shown to be chromatographically identical with 11β-hydroxyandrosterone in a mixed chromatogram.

graphed in the L.-P.G. system (72 hours). Four zones were visualized by the Zimmermann reagent (4-I, 4-II, 4-III and 4-IV, Table 2). Following elution and crystallization from acetone, zone 4-I yielded 1.0 mg. of crystals melting at 200–228°. Admixture of an authentic sample of etiocholane-3α,11β-diol-17-one (m.p. 234–236°) did not cause a depression in the melting

point of the crystals. The infra-red spectrum agreed with that of etiocholane-3α,11β-diol-17-one. The mother liquors contained a material which gave a brownish-purple Zimmermann reaction and an orange dinitrophenylhydrazone on paper and had an absorption maximum at 275 mμ. This substance was present in insufficient quantity for identification. Zones 4-II, 4-III and 4-IV had the mobility of 11β-hydroxyandrosterone, etiocholan-3α-ol-11,17-dione and 11-ketoandrosterone, respectively. In a mixed chromatogram with an authentic sample, zone 4-II was proved to be chromatographically identical with 11β-hydroxyandrosterone. The eluate of zone 4-III was identified by infra-red spectrometry as etiocholan-3α-ol-11,17-dione. However, the material contained in zone 4-II was too impure for further identification. The material which migrated faster than zone 4-IV and the "run off" fraction was rechromatographed in the L.-P.G. system (24 hours) and a single zone was detected by the Zimmermann reagent (5-I, Table 2). Zone 5-I had the mobility of etiocholan-3α-ol-17-one and was identified as such by infra-red spectrometry.

"Hot Hydrolysis" Fraction.—The neutral ketonic material was chromatographed in the L.-P.G. system (24 hours). Five zones which gave colors with the Zimmermann reagent were detected (6-I, 6-II, 6-III, 6-IV and 6-V, Table 2). Zone 6-I had the mobility of etiocholan-3α-ol-11,17-dione and was identified as such by infra-red spectrometry. Zones 6-II and 6-III gave brownish-purple colors with the Zimmermann reaction and yellow dinitrophenyl-hydrazones on paper. The eluates of these zones had no absorption maxima in the 240 mμ region, were non-crystalline and no identification was achieved. Zone 6-IV (Table 2) had the mobility of $\Delta^{9(11)}$-etiocholen-3α-ol-17-one and was identified by its infra-red spectrum. Zone 6-V had the mobility of $\Delta^{9(11)}$-androsten-3α-ol-17-one. One milligram of crystals melting at 169-180° was obtained by crystallization from methanol-water. The infrared spectrum of the crystals was identical with that of authentic Δ^{9}-androsten-3α-ol-17-one.

Patient No. 2

A 59 year old woman with rheumatoid arthritis received 1 gm. of 21-desoxycortisone orally in daily doses of 100 mg. for 10 days. The urine sample which was subjected to isolation studies represented 970 mg. of administered 21-desoxycortisone.

Only the "hot hydrolysis" fraction of the three day control urine was available. The eluates of the zones detected by the Zimmermann reagent on paper were both impure and small in quantity, making identification impossible.

The experimental urine yielded 6 mg. of crude pregnane-3α-17α-diol-

11,20-dione which was isolated in the manner described in the experimental section of patient No. 1. Crystallization from acetone yielded 1 mg. of crystals (m.p. 195–198°) which were identified by mixed melting point and infra-red analysis. In addition to the pregnane derivative, etiocholan-3α-ol-11,17-dione was isolated and identified by its infra-red spectrum. No etiocholan-3α,11β-diol-17-one was found, and the quantities of excreted $\Delta^{9(11)}$-etiocholen-3α-ol-17-one (0.4 mg.) and $\Delta^{9(11)}$-androsten-3α-ol-17-one (0.75 mg.) (which were identified by infra-red spectrometry) did not permit the conclusion that these steroids are metabolites of 21-desoxycortisone in this patient. As with the experimental urine of patient No. 1, etiocholan-3α-ol-17-one (3.8 mg.) was isolated and identified by infra-red analysis. However, no androsterone was found.

DISCUSSION

The results of the isolation studies of pretreatment control urines and 21-desoxycortisone urine of patient No. 1 are summarized in Table 3. Pregnane-3α,17α-diol-11,20-dione is a urinary metabolite of 21-desoxycortisone and no corresponding substance was obtained from the control

TABLE 3. STEROIDS ISOLATED IN 21-DESOXYCORTISONE METABOLISM STUDY
(Patient No. 1)

Steroid	Amounts isolated (crude) mg.	
	Control period[1]	Administration period
Pregnane-3α,17α-diol-11,20-dione	absent	7.1 (0.40%)[2]
Etiocholane-3α,11β-diol-17-one	0.32	7.6 (0.42%)
$\Delta^{9(11)}$-Etiocholen-3α-ol-17-one		
11β-Hydroxyandrosterone	5.1	3.1 (0.17% ?)
$\Delta^{9(11)}$-Androsten-3α-ol-17-one		
Etiocholan-3α-ol-11,17-dione	0.6	4.9 (0.27%)
Etiocholan-3α-ol-17-one	3.8	3.5

[1] Quantities listed are corrected to the equivalent of 18 days urine collection (the 21-desoxycortisone administration period); calculations are based on the maximum amounts of crude material found.

[2] The value in parenthesis is the amount of crude material isolated as per cent of administered 21-desoxycortisone (2.35 gm.)

urine. 21-Desoxycortisone was converted to pregnane-3α,17α-diol-11,20-dione to the extent of 0.4% in patient No. 1 and to 0.62% in patient No. 2. This conversion occurred to a lesser extent than was observed for the corresponding conversion of hydrocortisone[1] and cortisone[2] to pregnane-3α,17α,21-triol-11,20-dione.

No unchanged 21-desoxycortisone was detected in the urines of both patients, although significant quantities of hydrocortisone[1] and cortisone[2] were isolated following the administration of these steroids.

Etiocholane-3α,11β-diol-17-one and $\Delta^{9(11)}$-etiocholen-3α-ol-17-one (which

arose during the hot acid hydrolysis) and etiocholan-3α-ol-11,17-dione are considered to be metabolites of the administered 21-desoxycortisone in patient No. 1. However, a much smaller yield of etiocholane-3α,11β-diol-17-one and etiocholan-3α-ol-11,17-dione was obtained from 21-desoxycortisone than after oral administration of hydrocortisone[1] and cortisone.[2]

It is not possible from the present study to conclude that 11β-hydroxyandrosterone is a metabolite of 21-desoxycortisone (Table 3).

In contrast to the results obtained following the administration of hydrocortisone[1] and cortisone,[2] 21-desoxycortisone does not suppress the excretion of etiocholan-3α-ol-17-one. Patient No. 1 did not excrete androsterone during the control period nor during the 21-desoxycortisone period and it is not possible from this study to conclude whether 21-desoxycortisone has any effect on the secretion of androsterone precursors.

The isolation of pregnane-3α,17α-diol-11,20-dione, etiocholane-3α,11β-diol-17-one and etiocholan-3α-ol-11,17-dione, following the administration of 21-desoxycortisone, is in accord with other findings with respect to the conversion of C-21 steroids possessing an oxygenated ethyl side chain to ring A saturated C-21 and C-19 metabolites of the 5β configuration.

SUMMARY

1. A total of 3 gm. and 1 gm., respectively, of 21-desoxycortisone (Δ^4-pregnen-17α-ol-3,11,20-trione) have been administered to two female patients with rheumatoid arthritis.

2. Pregnane-3α,17α-diol-11,20-dione has been isolated and identified as a metabolite of 21-desoxycortisone by melting point and infra-red spectrometry in the urine of both patients.

3. Etiocholane-3α,11β-diol-17-one (and its dehydration product $\Delta^{9(11)}$-etiocholen-3α-ol-17-one) and etiocholan-3α-ol-11,17-dione have been isolated and identified and are considered to be metabolites of 21-desoxycortisone from the urine of one patient.

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EFFECTS OF HYPOTHALAMIC LESIONS AND SUBSEQUENT PROPYLTHIOURACIL TREATMENT ON PITUITARY STRUCTURE AND FUNCTION IN THE RAT[1]

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INTRODUCTION

THERE is increasing evidence that many phases of anterior pituitary activity can be impaired by damaging either the hypothalamus or its neural or vascular connections with the hypophysis (Harris, 1948; Greer, 1952; Long, 1952). Our investigations were designed to relate functional alterations of the adenohypophysis produced in this way to concomitant morphological changes of the gland, the latter being examined by recently devised staining techniques which have considerably clarified the histophysiology of the pituitary in the rat (Purves and Griesbach, 1951a and b Halmi, 1952a).

We chose the use of hypothalamic lesions as a method of disrupting hypothalamic-hypophysial relationships since it presented two distinct advantages over infundibular stalk section. Firstly, it permitted localized destruction within the hypothalamus which we hoped might result in discrete effects on pituitary functions, and secondly, we believed that it would not be subject to the criticisms levelled at infundibular stalk section, a) that regeneration of the portal vessels may occur, nullifying the effect of the intervention (Harris, 1948), or b) that the operation may impair the nutritive vascular supply of the hypophysis (Barrnett and Greep, 1951a).

In the present study our primary interest was directed at the thyroid stimulating function of the anterior pituitary, which Greer (1951) recently showed to be impaired by hypothalamic lesions. We followed Greer's approach in treating the rats with propylthiouracil, since interference with

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thyrotrophin secretion is more readily discerned when this function is stimulated by the administration of a goitrogen. Although in the majority of our animals only the morphological evidence of thyroid stimulation was examined, in a small series of rats both the morphology and the iodide concentrating power of this gland were studied. Because the hypothalamic lesions employed produced a variety of effects in addition to that on thyrotrophin secretion, observations made on the intermediate and posterior pituitary lobes, gonads, adrenals, body habitus and temperament of these rats have also been included in this report.

MATERIALS AND METHODS

Bilaterally symmetrical hypothalamic lesions were made in male rats of both Sprague-Dawley and Long-Evans strains (initial weights from 215 to 360 g.) by electrolysis with a direct current of 2–2.5 milliamperes for 20–30 second. The lesions were placed in various positions within the hypothalamus by means of either a modified Horsley-Clarke stereotaxic apparatus or the Krieg-Johnson stereotaxic instrument for rats. Skull markings were used to locate the coagulating electrode in the vertical plane and the tip of the electrode was withdrawn 1/2–1 mm. from the floor of the cranial cavity by way of horizontal orientation. Nembutal anesthesia was used with the Horsley-Clarke apparatus, ether with the Krieg-Johnson machine.

Beginning after several days of recovery from the operation, 50 rats were injected daily for 15 to 21 days with 20 mg. of propylthiouracil (PTU) which was suspended in water with powdered gum acacia. Six rats (S series) received a weaker goitrogenic stimulus consisting of 10 mg. of PTU solution (in dilute NaOH) injected daily for 10 days. Although animals with non-effective lesions served as operated controls, 8 were maintained on the stronger goitrogenic regimen as non-operated controls. All rats were allowed Purina chow and tap water *ad libitum*.

At the termination of the PTU injections (between 23 and 43 days postoperatively), 45 of the 56 lesioned rats and 4 unoperated controls, which were used for morphological studies only, were sacrificed with ether. The following organs were weighed on a torsion balance: thyroids, adrenals, testes, seminal vesicles, and ventral prostates. Thyroids and testes were fixed in SUSA for histological examination. Pituitaries were fixed immediately in SUSA-picric acid (9:1) without being weighed, and brains were fixed in 10% formalin.

The 11 lesioned and 4 unoperated rats used for studies of thyroid iodide concentrating power (S, R-H, and W series) were anesthetized with ether and killed by exsanguination (23–41 days postoperatively) after the administration of PTU and carrier-free radioiodide according to the method of Vanderlaan and Greer (1950). One lobe of the thyroid and an aliquot of serum were used for the determination of the thryoid:serum iodide concentration ratio (T/S),while the other lobe of the gland and the rest of the organs, which were saved for histological examination, were treated as described above.

All tissues were embedded in paraffin and 6 μ sections of testis and thyroid were stained by Gomori's trichrome method (1950). Pituitaries were sectioned in the horizontal plane at 4 μ and 6 μ. Representative sections at several levels were mounted together, and the 4 μ sections were stained both with Gomori's trichrome and with the aldehyde-fuchsin (AF)-trichrome method of Halmi (1952b), while the 6 μ sections were stained with the periodic acid-Schiff (PAS) technique for glycoproteins (Purves and Griesbach,

1951a). Combined use of these techniques permits the identification of the thyrotrophic basophils (beta cells) which stain with both AF and PAS, and of the gonadotrophic basophils which are AF negative and PAS positive (Purves and Griesbach, 1951b, c; Halmi, 1952a). The former will be referred to as "thyrotrophs," the latter as "gonadotrophs," according to the terminology of Purves and Griesbach (1951a). "Thyroidectomy cells" will be the term applied to the AF and PAS negative, hypertrophic, vacuolated thyrotrophs, which are characteristic of thyroid hormone deficiency states.

Blocks of the brains were sectioned in the coronal plane at 20 μ, every fifth and sixth section between the preoptic region and the mammillary body being mounted and stained with thionine for localization of the lesions.

RESULTS

Thyroid

In 10 of the 50 lesioned rats on the stronger goitrogenic regimen (20 mg. of propylthiouracil (PTU) per day for a period ranging from 15 to 21 days) the thyroids weighed 25 mg. or less. The mean weight of glands from unoperated controls receiving this treatment was 71 mg. (range: 46–101 mg.). Since the thyroids of the remaining 40 lesioned rats varied in weight between 25 mg. and 101 mg., it is obvious that the response to PTU of some of these glands was partially impaired. We have arbitrarily classed 6 glands weighing between 25 and 40 mg. as exhibiting such a partially impaired response. (The expression of organ weights in absolute values is more meaningful than their expression on a body weight basis, owing to the occurrence of hypothalamic obesity in a number of the animals.)

For convenience the terms "goiter block" and "partial goiter block" have been used to designate the condition of those rats whose thyroids weighed less than 25 mg., and between 25 and 40 mg., respectively. Histologically the thyroids of rats with "goiter block" were similar to or only slightly more stimulated than "normal" thyroids from rats in this laboratory (Fig. 1), with cuboidal to low columnar acinar epithelium surrounding moderately dense or somewhat dilute and partly acidophilic colloid. The thyroids of all other lesioned rats (including those with "partial goiter block") were histologically similar to those of the goitrogen treated controls (Fig. 2), with tall columnar epithelium lining hyperplastic follicles virtually devoid of colloid.

The microscopic appearance of the thyroids in the S series of 6 rats (which received the weaker goitrogenic stimulus) seemed to be a more useful criterion of the degree of thyrotrophic stimulation than the gross size of the gland. The data for this group, including the thyroid:serum iodide concentration ratio values, are summarized in Table 1. Histologically the thyroids of S0 and S4 were clearly hyperplastic (Fig. 4), in S6 and S7 the goitrous response was partially blocked, while S2 and S5 showed no excessive stimulation (Fig. 3) There was no correlation between morphologi-

cal appearance and iodide concentrating capacity of these thyroids.

In the Rh and W series (receiving the PTU suspension for 16 days) 4 unoperated rats had thyroid:serum iodide concentration ratios (T/S) between 164:1 and 414:1, while 3 lesioned rats with "goiter block" and 2 with goitrous thyroids showed T/S values of 202:1, 430:1, 275:1 and 215:1 and 251:1, respectively.

Since the T/S for untreated rats is approximately 25:1 (Vanderlaan and Vanderlaan, 1947; Halmi *et al.*, 1953), the data on the S, Rh and W rats confirm the finding of Greer (1951) that the T/S may be as markedly

TABLE 1. THYROID WEIGHTS AND THYROID:SERUM IODIDE CONCENTRATION RATIOS (T/S) IN LESIONED RATS RECEIVING 10 MG. PROPYLTHIOURACIL SOLUTION PER DAY FOR 10 DAYS

Rat No.	S0	S4	S6	S7	S2	S5
Body weight (gm.)	195	165	295	255	192	275
Thyroid weight (mg.)	31.6	19.8	9.6	11.6	8.0	7.2
T/S	111:1	76:1	156:1	212:1	119:1	72:1
Histological appearance of thyroid	Markedly stimulated (Fig. 4)		Moderately stimulated		Unstimulated (Fig. 3)	

increased by PTU in rats with hypothalamic lesions as in unoperated controls, even though the lesions have prevented a commensurate degree of thyroid hyperplasia from developing.

Gonads

The testicular weights of 27 of the 56 lesioned rats in this study were decreased to less than 2 gm. Of these, 14 weighed less than 900 mg., with a minimum of 520 mg. Since in 22 lesioned and 8 unoperated rats on the goitrogenic regimen the testes were of normal histologic appearance (Fig. 5) and weighed over 2500 mg. (range: 2545–3864 mg.), the gonad atrophy must be attributed to the effect of specific lesions, and not to the PTU or to non-specific damage caused by the hypothalamic intervention.

The gonad atrophy was of two types. In a few rats there was sloughing of spermatogenic epithelium, many tubules containing Sertoli cells only, while there were numerous normal appearing Leydig cells in between the tubules (Fig. 7). The prostates and seminal vesicles of these animals were as well maintained as those of the controls. This picture is identical with that found in cryptorchidism.

In the majority of cases with testicular atrophy, however, the tubules showed spermatogenic arrest (no forms present more mature than secondary spermatocytes) and the interstitial cells were of the deficiency type.

TABLE 2. MEAN TESTICULAR AND ACCESSORY SEX GLAND WEIGHTS IN RATS WITH HYPOTHALAMIC LESIONS AND IN UNOPERATED CONTROLS

		Lesioned			*Unoperated*
Number of rats		14	20	22	8
Testis	Range	520–900	900–2500	2500–3879	2904–3423
	Mean	736	1646	3184	3200
Ventral prostate	Mean	52	106	303	251
Seminal vesicle	Mean	105	177	346	288

This type of atrophy is similar to, and in the most pronounced cases was indistinguishable from, that seen after hypophysectomy (Fig. 6). There was regression of the accessory sex glands in these animals which roughly paralleled that of the testes (Table 2). The accessory weights of both lesioned and unoperated rats with gonads of normal size were somewhat lower than those of untreated rats of the same age, suggesting that the goitrogen may have had some effect on androgen production, without producing testicular atrophy.

Combinations of cryptorchid-like and hypophysectomy-like gonadal atrophy occurred. The testes of many of the lesioned rats were actually found (both at autopsy and by palpation during the postoperative period) to be withdrawn into the abdominal cavity.

Adrenals

Adrenal weights varying from 30 to 59 mg. were observed in the unoperated controls of this series. The adrenals of the lesioned rats weighed between 19 and 60 mg. Although the occurrence, in four rats, of adrenals weighing less than 30 mg. suggests that partial adrenal atrophy may have resulted from certain lesions, the present data do not warrant a definite conclusion in view of the great variability of adrenal size.

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EXPLANATION OF PLATE 1

FIG. 1. Thyroid (wt. 13 mg.) of rat with lesion preventing enlargement in response to propylthiouracil (20 mg./day for 18 days). Acinar epithelium predominantly low columnar. Many follicles contain acidophilic colloid. ×210.

FIG. 2. Enlarged (55 mg.) thyroid of unoperated control rat following propylthiouracil treatment (20 mg./day for 14 days). The gland is hyperemic. Follicles devoid of colloid and lined by high columnar epithelium. ×210.

FIG. 3. Thyroid of rat S5 (wt. 7.2 mg.). No hyperplastic response to propylthiouracil (10 mg./day for 10 days). Cuboidal acinar epithelium enclosing dense colloid. Thyroid:serum iodide concentration ratio (T/S) = 72:1. ×210.

FIG. 4. Hyperplastic thyroid of rat S4 (wt. 19.8 mg.) which received the same goitrogenic treatment as S5. Follicles, lined with columnar epithelium, contain only a little dilute colloid. Note numerous mitoses (arrows). T/S = 76:1. ×210.

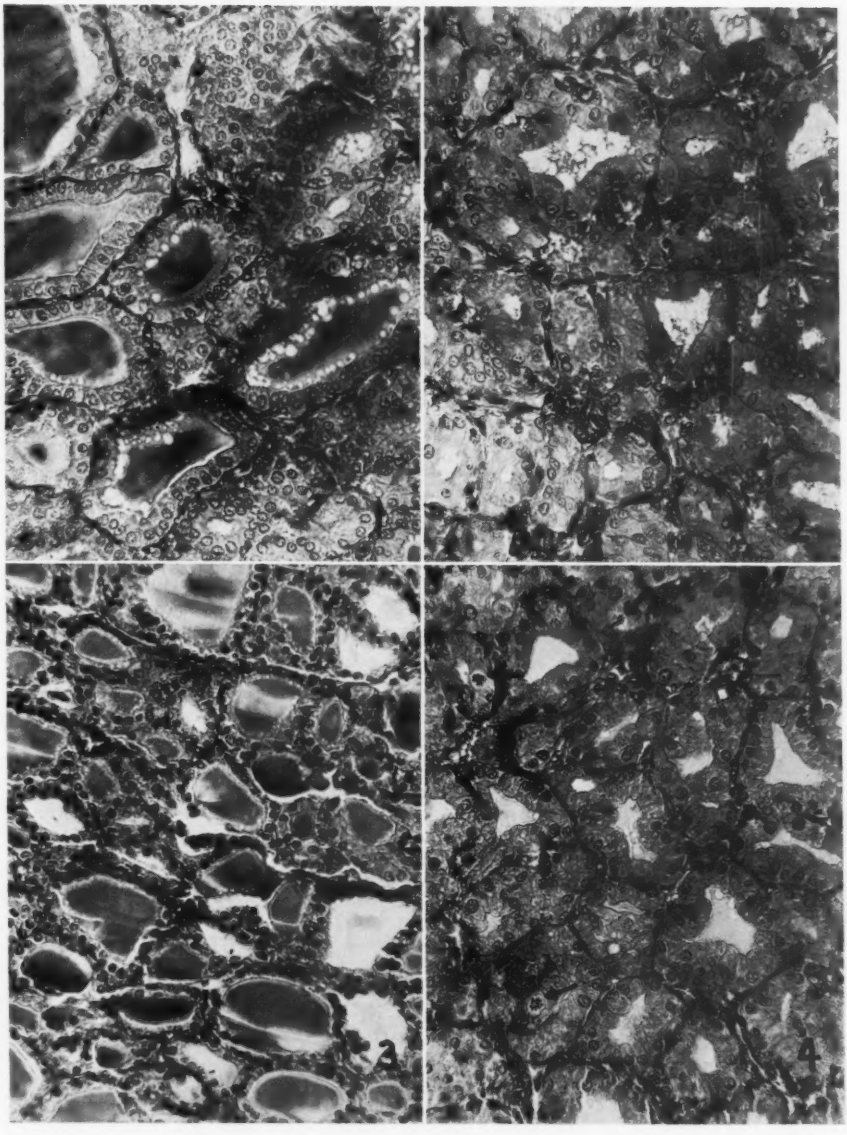

PLATE 1

Pituitary

Anterior lobe. The aldehyde-fuchsin (AF) staining granulation of the thyrotrophs was virtually or completely absent from every pituitary. Numerous thyroidectomy cells were present in the anterior lobes of the controls and of most of the lesioned rats with enlarged thyroids (Fig. 8). These cells were either completely absent or very sparse in the pituitaries of rats with the most severe "goiter block" (Fig. 9). In the rats whose thyroid hyperplastic response was less markedly impaired, but yet sufficiently so to be classed as "goiter block" (<25 mg.), the thyroidectomy cells were definitely less numerous than in the controls. Among the 6 rats judged to have "partial goiter block," only 3 showed obviously fewer thyroidectomy cells than the PTU controls. On the other hand, there were lesioned rats in which the thyroidectomy cells were considerably less abundant than in the controls even though their thyroids were markedly hyperplastic (>40 mg.).

In both the unoperated rats and those operated animals which did not show gonadal atrophy, the gonadotrophs appeared normal although possibly less numerous than in rats not treated with PTU (Fig. 10). They were as well maintained (or even slightly enlarged) in the pituitaries of rats with purely cryptorchid-like testicular changes. However, where severe hypophysectomy-like gonadal regression had occurred, the gonadotrophs were either totally absent from the pituitaries or so reduced in size and PAS positive granulation as to be barely distinguishable (Fig. 11). In those cases in which this type of testicular atrophy was of lesser extent, the degree of maintenance of these cells roughly paralleled that of the testes and accessories.

The acidophils were almost completely degranulated in every rat which had received the stronger goitrogenic treatment. In many of the lesioned animals these cells, in addition to being degranulated, were found to be shrunken, with pyknotic nuclei and cytoplasmic vacuolation. Degranulation of the acidophils was less extensive in the pituitaries of the S series of rats, to which the weaker goitrogenic stimulus had been administered.

Although in order to obtain optimal preservation the pituitaries were

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EXPLANATION OF PLATE 2

FIG. 5. Normal testis from rat with hypothalamic lesion. (Wt. 3160 mg.) ×120.

FIG. 6. Hypophysectomy-like testicular atrophy in lesioned rat. Greatly decreased tubular diameter. No spermatids or sperm present. (Wt. 575 mg.) ×120.

FIG. 7. Cryptorchid-like testicular atrophy in lesioned rat. Tubular diameter decreased. Sloughing of spermatogenic epithelium, many tubules with Sertoli cells only. Leydig cells appear normal. (Wt. 1008 mg.). ×120.

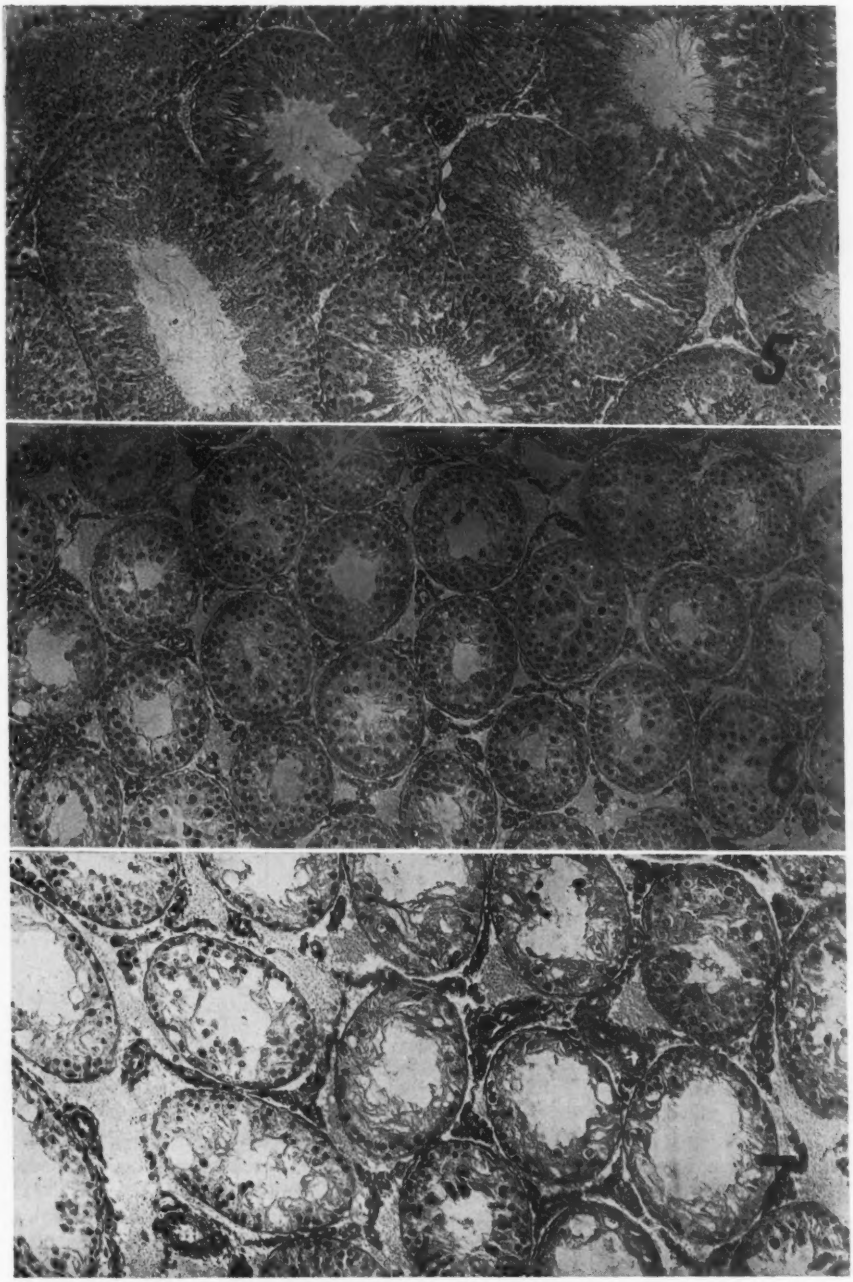

PLATE 2

fixed without being weighed, many of the glands of lesioned rats appeared to be distinctly smaller than those of the controls. This was probably due, in large part, to shrinkage of the acidophils.

Occasionally, small areas of infarction were noted in these hypophyses, but even the largest of the infarcts did not appear to involve more than one-fourth of the volume of the anterior lobe. On the other hand, many of the glands appeared to be hyperemic, the sinusoids being dilated and engorged with blood.

Posterior lobe. In 25 of the 56 lesioned rats the posterior lobes were atrophic. Microscopically these showed increased cellularity and depletion of the normally present AF staining intercellular material which we believe to be identical with the chrome-alum hematoxylin stainable neurosecretory substance described by Bargmann and Scharrer (1951). Urinary output was not measured, and consequently we do not know whether these animals suffered from diabetes insipidus.

Intermediate lobe. In the pituitaries of several hundred rats without hypothalamic lesions thus far examined, including a score or more injected chronically with PTU, the intermediate lobes have been comprised of uniform cells (Fig. 12) with more or less AF positive cytoplasmic granulation. Mitoses were never encountered. In 18 of the 56 lesioned rats used in this study, the intermediate lobes appeared enlarged (although no actual measurements of size were carried out) and showed increased lobularity, considerable nuclear pleomorphism, many large vacuoles and numerous mitoses (Fig. 13). The cytoplasm of the enlarged cells did not stain with AF. In many cases the intermediate lobe appeared to be invading the atrophic posterior lobe. In at least one case a well maintained posterior lobe was coexistent with a hyperplastic pars intermedia (Fig. 14).

Most of the vacuoles found in these abnormal intermediate lobes seemed to be the result of degeneration of dividing cells in which vacuolation of

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EXPLANATION OF PLATE 3

FIG. 8. Anterior pituitary of lesioned rat with propylthiouracil induced thyroid enlargement. Note abundance of thyroidectomy cells (arrows). Trichrome; ×340.

FIG. 9. Anterior pituitary of rat in which hypothalamic lesion prevented thyroid enlargement following propylthiouracil treatment. Thyroidectomy cells absent. Note numerous erythrocytes in sinusoids. Trichrome, ×340.

FIG. 10. Anterior pituitary of lesioned rat with normal gonads. Region adjacent to residual lumen. Intermediate lobe at bottom of field. Characteristic clustering of gonadotrophs around the portal vessels. PAS, ×200.

FIG. 11. Section from lesioned rat with hypophysectomy-like testicular atrophy. Same region of pituitary as shown in Fig. 10. Gonadotrophs cannot be identified. PAS, ×200.

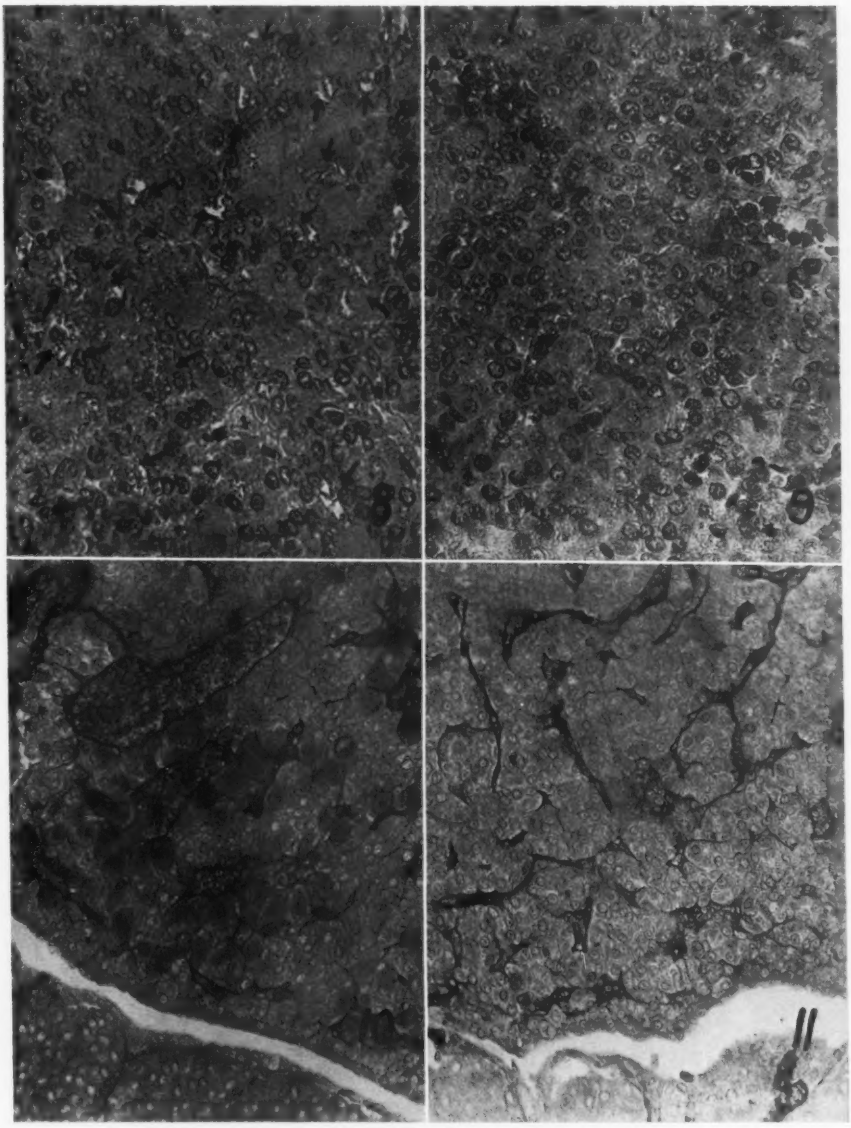

PLATE 3

the cytoplasm accompanied disintegration of the mitotic figure (Fig. 16) or clumping of the chromosomes (Fig. 17). However, many of the cells showed apparently normal mitotic figures (Fig. 15). Occasionally, cytoplasmic vacuolation was seen to arise in non-dividing cells (Fig. 18).

Other Effects

A large number of the lesioned animals became both markedly obese and savage, frequently exhibiting hyperphagia and belligerence even before the effects of the anesthesia had fully worn off. Since the controls invariably lost weight during the period of goitrogen administration, mere maintenance of weight in the lesioned rat receiving PTU was indicative of mild obesity. Some of the lesioned animals gained considerable weight in spite of the antithyroid treatment. Occasionally a rat became fat but remained quite tractable, while some of the savage rats never developed obesity.

On the other hand, some of the rats with lesions placed in the anterior region of the hypothalamus did not eat at all, lost weight rapidly, and died. Still other animals with lesions in the same general location succumbed owing to difficulties in thermostasis (hyperthermia). These lesions were made in virtually the same sites as those which produced "goiter block" in surviving animals, and indeed, many of the rats which failed to survive the experimental period had small thyroids at postmortem examination. Since autolysis was usually several hours advanced by the time autopsy was performed, the endocrine tissues were worthless for histological examination and data were not collected on these animals.

Correlation of effects and localization of lesions

Although the full syndrome of effects ("goiter block," gonad atrophy, intermediate lobe hyperplasia, posterior lobe atrophy, obesity and ferocity) frequently occurred in the same animal and many of these effects were repeatedly observed together, we were able to demonstrate by cross tabulation of our findings that most of them were mutually independent. There was a tendency to correlation of effects in the following cases:

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EXPLANATION OF PLATE 4

FIG. 12. Normal pars intermedia. Note uniformity of nuclei. Trichrome. ×375.

FIG. 13. Hyperplastic intermediate lobe of lesioned rat. Note nuclear pleomorphism, mitosis (M), and two vacuoles (V). Trichrome, ×375.

FIG. 14. Hyperplastic intermediate lobe adjacent to normal posterior lobe (upper part of field). Note nuclear pleomorphism and several vacuoles (arrows) in pars intermedia. The dark droplets in the neurohypophysis are aldehyde-fuchsin stained neurosecretory material. AF-trichrome, ×280.

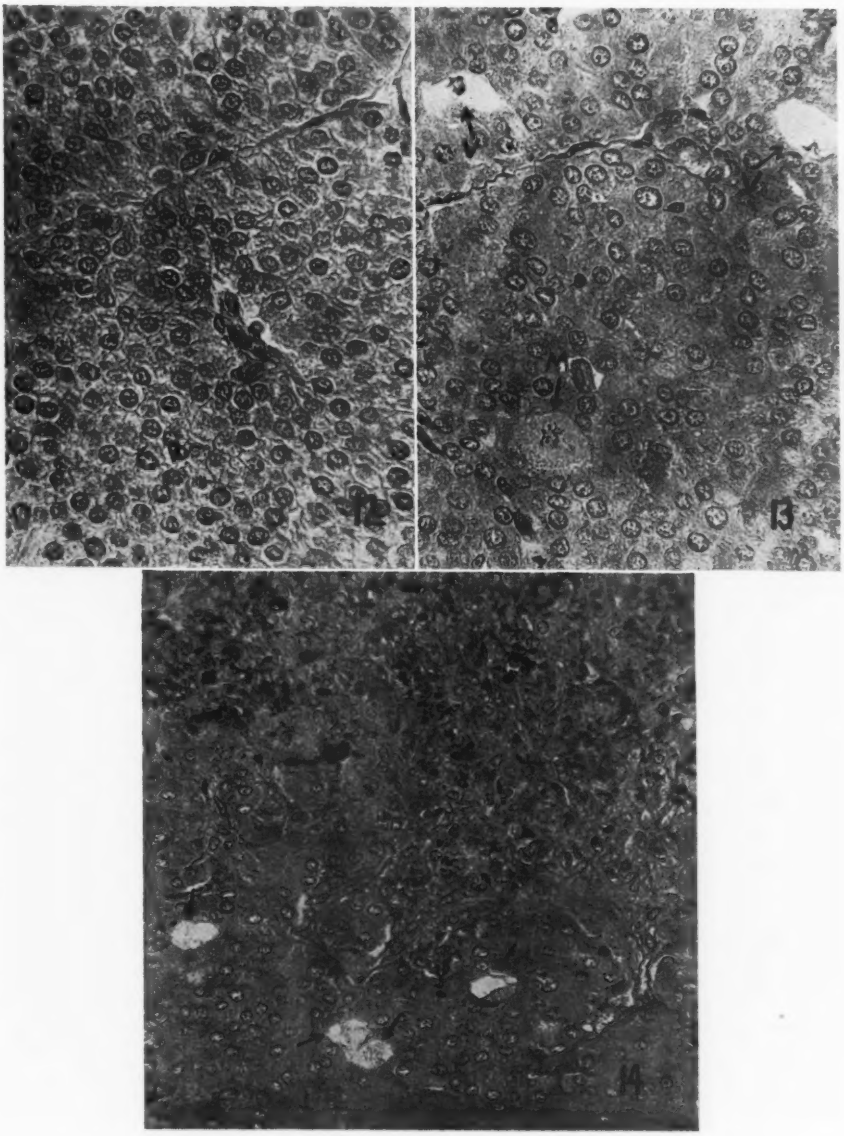

PLATE 4

1) "Goiter block" was usually accompanied by changes in the pars intermedia and atrophy of the posterior pituitary. Extremes of this condition never occurred in the absence of intermediate lobe hyperplasia, although "partial goiter block" did. The intermediate lobe changes were occasionally found without "goiter block," but in these pituitaries there was usually some decrease in thyroidectomy cells below the expected number. Posterior lobe atrophy frequently developed alone.

2) Hypophysectomy-like gonad atrophy, obesity and ferocity generally occurred together but were clearly separable.

Detailed analysis of the lesions is beyond the scope of this paper. Nevertheless, it may be stated that the effects grouped under 1) above resulted from hypothalamic lesions which were basal, close to the midline and extended anteriorly to encroach upon the optic chiasm; and that those phenomena grouped under 2) followed the placing of lesions which were likewise basal, fused in the midline, and which extended from the anterior margin of the median eminence to the premammillary nuclei. The lesions which produced the complete syndrome were very extensive, involving both of these regions.

Although the rather large lesions employed only rarely allowed separation of the effects gathered within these two subgroups, separation of the subgroups from each other was found more commonly, providing clear demonstration that the thyrotrophic and gonadotrophic functions of the pituitary can be selectively impaired by appropriate hypothalamic lesions.

DISCUSSION

The impairment, by hypothalamic lesions, of the hyperplastic response of the thyroid to goitrogen, without a simultaneous interference with the propylthiouracil (PTU) induced elevation of the thyroid:serum iodide concentration ratio (T/S), is in agreement with the observations first made by Greer (1951). Greer (1952), to explain this dichotomy between the morphological and functional responses of the thyroid to PTU, hypothesized that a second TSH, not under neural control, stimulates the activity of the thyroid iodide trap.

However, we believe that another explanation is available. PTU has

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EXPLANATION OF PLATE 5

FIG 15. Two dividing cells (metaphase) and a large vacuole. AF-trichrome, ×1000.

FIG. 16. Disintegration of mitotic figure with vacuolar transformation of cytoplasm. Trichrome, ×1200.

FIG. 17. Two daughter cells with clumped chromosomes and vacuolated cytoplasm. Trichrome, ×1200.

FIG. 18. Multiple vacuoles in cytoplasm of non-dividing cell. Trichrome, ×1200.

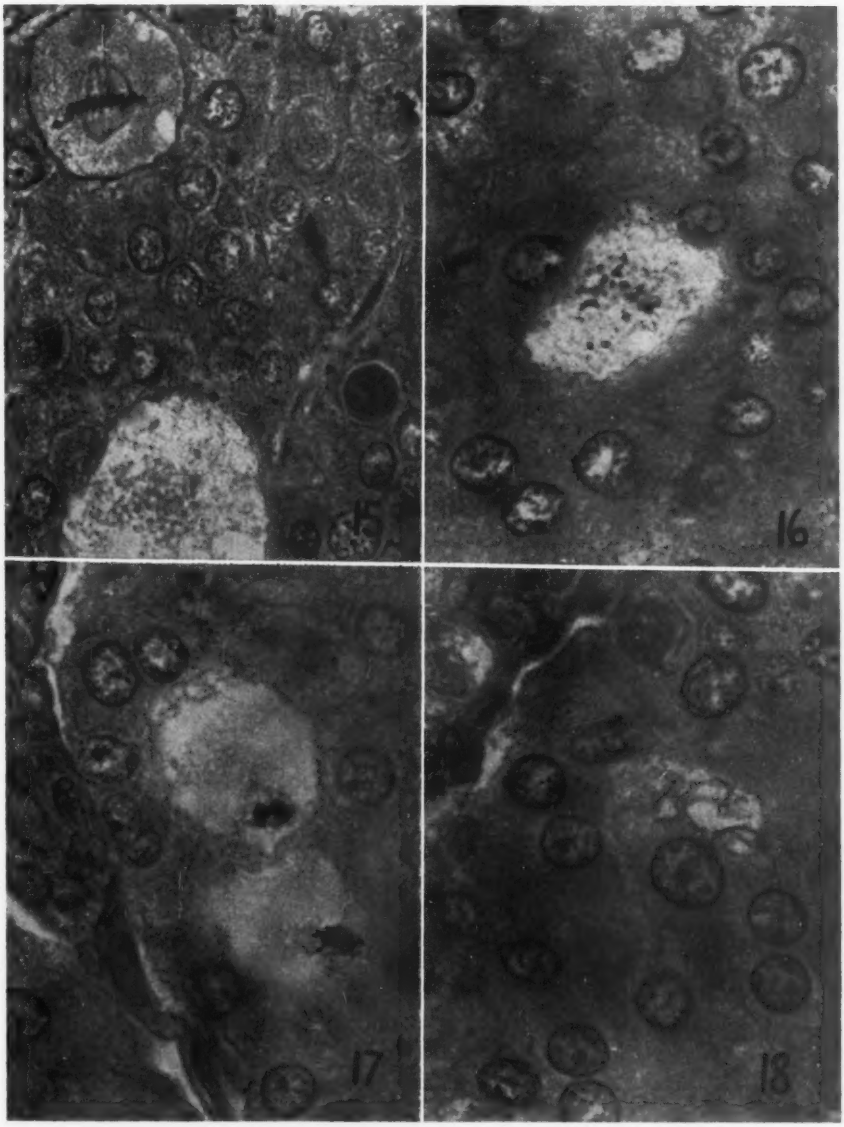

PLATE 5. Cytological changes in hyperplastic intermediate lobes.

been found to potentiate strongly the effect of TSH on the iodide concentrating ability of the thyroid without appreciably altering the morphological response of the gland to this hormone (Halmi *et al.*, 1953). Since the histological appearance of even the least stimulated thyroid in this study indicates that thyrotrophin secretion was not completely abolished by the hypothalamic lesions, the potentiation by PTU of the effect of this endogenous hormone on thyroidal iodide concentrating power could account for the combination of markedly elevated T/S values with little or no morphological evidence of excessive thyrotrophic stimulation.

Whether more extensive lesions would completely abolish the secretion of TSH, and with it the goitrogen induced elevation of the T/S, remains to be seen. However, since the lesions which impair TSH secretion are in that region of the hypothalamus in which the amount of destruction already employed has resulted in failure of half of the operated rats to survive the experimental period, it seems questionable that further impairment of TSH secretion could be effected with impunity by increasing the size of the lesions.

The changes which occur in the anterior pituitary of the rat in hypothyroidism have been interpreted as follows (Purves and Griesbach, 1951b, c; Halmi, 1952a): The early disappearance of AF staining granulation from the thyrotrophs can be correlated with a massive discharge of stored TSH. The thyroidectomy cells, which subsequently arise from the thyrotrophs, produce and emit large amounts of TSH without storing very much of this hormone. They are devoid of beta granules, which appear to be a morphological manifestation of TSH storage.

The virtual absence of beta granulation from the anterior pituitaries of all the rats in this study indicates that the hypothalamic lesions did not interfere with the initial precipitous discharge of thyrotrophin in response to the PTU induced hypothyroid condition. The observation that the animals which showed no thyroid hyperplasia also lacked thyroidectomy cells, while the latter were invariably abundant in the unoperated controls, is in keeping with the idea that the presence of thyroidectomy cells in the hypophysis is an indication of TSH hypersecretion. However, the scarcity of these cells in the pituitaries of some of the operated rats which displayed a striking thyroid hyperplastic response to the goitrogen calls attention to the fact that the thyroidectomy cell is not an indispensable manifestation of TSH hypersecretion. Previously, a marked hyperplastic response of the thyroid has been reported to occur in rats treated with estrogen plus thiouracil without any accompanying development of thyroidectomy cells in the pituitary (Desclin and Ermans, 1950).

The absence of acidophilic granulation from the hypophyses of all rats on the stronger goitrogenic regimen can be interpreted as a sign of complete

or almost complete block of thyroid hormone synthesis (Purves and Griesbach, 1947). The shrinkage, in addition to degranulation, of the acidophils in some of the lesioned rats suggests that the lesions themselves may have had an effect on these cells.

The parallelism between the degree of regression of the gonadotrophs on the one hand, and of hypophysectomy-like testicular atrophy on the other, is in agreement with earlier evidence (Purves and Griesbach, 1951a) that these are the cells which secrete gonadotrophin (probably both FSH and ICSH). The excellent maintenance of these cells in the pituitaries of rats with purely cryptorchid-like gonadal atrophy supports our belief that this type of regression was not secondary to lesion-induced hypophysial alterations, and was probably simply the sequel of the observed withdrawal of the testes from the scrotum. Such withdrawal has been reported previously to occur in rats with hypothalamic lesions leading to obesity (Hetherington and Ranson, 1940).

Since certain lesions produced complete regression of the gonadotr phs without impairing the development of thyroidectomy cells while others resulted in the complete absence of thyroidectomy cells but had no effect on the gonadotrophs, the histological appearance of the pituitary provides evidence, corollary to that offered by the target organs, that hypothalamic lesions can selectively interfere with the secretion of thyrotrophin and gonadotrophin.

Real or apparent growth of the intermediate lobe of the mammalian pituitary following damage either to the hypothalamus or to the connections of the latter with the hypophysis has been noted in a variety of species: rat (Desclin, 1942), mouse (May and Stutinsky, 1947), rabbit (Brooks 1938), and dog (Stutinsky *et al.*, 1950). Desclin, believing this enlargement to result entirely from cellular hypertrophy (without hyperplasia), hypothesized that the intermediate lobe cells were engorged because they were unable to discharge their secretion into the adjacent posterior lobe which had atrophied following stalk section. This hypothesis is untenable because 1) there is no evidence that the intermediate lobe discharges its secretion into the posterior lobe, 2) the enlargement of the intermediate lobe involves mitotic divisions (Figs. 15, 16, 17) as well as simple cellular hypertrophy, and 3) posterior lobe atrophy is not necessary for the development of these changes (Fig. 14).

We are inclined to believe that the mammalian intermediate lobe changes are basically similar to those which occur in tadpoles (Etkin, 1941, 1943), where infundibular stalk section or transplantation of the hypophysis results in a spectacular hyperplasia of the pars intermedia. In Etkin's experiments pigmentation changes demonstrated unequivocally that the proliferating intermediate lobe tissue was also hypersecretory. The functional

state of the hyperplastic intermediate lobe of the rat is less readily determined than that of the tadpole and requires investigation. The significance of a possible hypersecretion of intermedin in the rat must of course await clarification of the role of this hormone in mammalian physiology.

It is a point of considerable interest that the nervous system appears to restrain the intermediate lobe, an influence opposite in direction to those it brings to bear upon the other two lobes of the hypophysis.

The possibility that the various alterations of anterior lobe function were the result of circulatory derangement should be considered. The panhypopituitarism which Greep and Barrnett (1951) and Barrnett and Greep (1951a and b) observed in stalk-sectioned rats could be fully accounted for by the extensive infarction of the anterior lobe which developed after this operation. In the present study varying degrees of functional and morphological alterations of the adenohypophysis were produced by hypothalamic lesions, but pituitary infarcts were infrequent and small and could not have been responsible for the findings. On the other hand, the dilatation of the sinusoids in many of these glands might possibly have been a sign of another sort of circulatory disturbance, such as stasis. Even if this were so, however, it is inconceivable that stasis could account for the selective impairment of thyrotrophin or gonadotrophin secretion which was observed.

This selectivity in the endocrine effects of hypothalamic lesions is perhaps the most significant of our findings, in that it militates strongly against the assumption that the infundibular stalk is of importance to the anterior lobe only by virtue of the nutritive vessels which it bears. Furthermore, it indicates the influence of relatively discrete hypothalamic regions on individual adenohypophysial mechanisms. Although the gonadotrophic and thyrotrophic basophils have distinctly different distributions within the anterior lobe (Purves and Greisbach, 1951a), they are often intermingled, and it is reasonable to assume that they are not supplied by separate vascular systems. If thus the hypophysial portal vessels provide a final common pathway for the transport of neurohumoral substances which regulate the secretory functions of the anterior pituitary, as suggested by Harris (1948), it now appears necessary to postulate both the production of more than one neurohumor and the discrete location within the hypothalamus of the responsible neurons.

SUMMARY

1. Bilateral electrolytic lesions were placed in various hypothalamic regions of adult male rats which were subsequently treated with propylthiouracil (PTU) for periods ranging from 10 to 21 days.

2. In a number of these animals one or more of the following sequelae of the lesions, varying in severity, were observed to occur: a) impairment of the hyperplastic response of the thyroid to PTU but no concomitant interference with the goitrogen induced elevation of the thyroid:serum iodide concentration ratio, b) gonad atrophy of the hypophysectomy type with commensurate regression of the accessory sex glands, c) cryptorchid-like testicular alterations, d) hyperplastic changes of the intermediate hypophysial lobe, e) atrophy of the neurohypophysis, f) obesity, g) savage behavior.

3. The depletion of pituitary beta and acidophilic granulation in response to the hypothyroid condition was not interfered with by any of the lesions. Thyroidectomy cells were virtually absent from the hypophyses of rats which showed no thyroid hyperplasia, but were invariably numerous in PTU treated controls. They were occasionally sparse in the pituitaries of lesioned rats with frankly goitrous thyroids. The gonadotrophic basophils showed regression in rats with hypophysectomy-like gonad atrophy, but not in the animals exhibiting cryptorchid-like testicular changes.

4. The effects on thyroid, intermediate and posterior pituitary lobes tended to occur with lesions in the anterior hypothalamus, whereas gonad atrophy, obesity and ferocity resulted from lesions involving the region between the median eminence and the premammillary nuclei. The thyrotrophic and gonadotrophic mechanisms of the pituitary could be independently altered by damage to the appropriate portions of the hypothalamus.

5. The significance of these findings with regard to pituitary histophysiology and the nature of the neural influence upon the adenohypophysis is discussed.

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ALTERATIONS IN THE WHITE BLOOD CELL PICTURE IN NORMAL, HYPOPHYSECTOMIZED AND SPLENECTOMIZED GUINEA PIGS DURING MILD STRESS AND FOLLOWING EPINEPHRINE INJECTION

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EARLIER studies on the pituitary-adrenal axis in the guinea pig (Schweizer and Long, 1950) are being extended using the decrease in lymphocytes and eosinophils as an indication of adrenal cortical secretion (Dougherty and White, 1944; Recant, Hume, Forsham and Thorn, 1950). Although it is standard procedure to use unanaesthetized mice and rats in studies of changes in the blood cell picture, many of the reports on alterations in the white blood cell count of the guinea pig are based on the use of anaesthetized animals (Bacchus, Heiffer and Altszuler, 1952; Piliero and Gordon, (personal communication); Samter, Erickson and Kofoed, 1952). Since the response of the guinea pig to anaesthesia is known to be uneven and since the use of anaesthesia introduces an additional variable in any experiment, it seemed desirable to standardize the blood cell counting procedure for the unanaesthetized guinea pig.

In the preliminary experiments designed to standardize the method it was evident that this species does not respond in all respects like others described in the literature. Samter, Erickson and Kofoed (1952) report that the guinea pig is unsuitable for studies on ACTH action because it is more refractory than other forms to small amounts of this hormone. As will be shown below, the guinea pig also failed to give the typical eosinopenic response three hours after epinephrine injection. Furthermore, even with the greatest care in handling the animals, the mere sampling of blood caused alterations in the blood cell picture which indicate that it responds like the mouse (Speirs and Meyer, 1949) with a mild stress reaction. This reaction was utilized in an attempt to elucidate the mechanisms affecting alterations in the white blood cell count in the guinea pig.

EXPERIMENTAL PROCEDURE

The adult (400–550 grams) male and female guinea pigs used in these experiments were raised in our laboratory (temperature 70°–80° F.) on a diet of purina chow supplemented daily by ample amounts of greens and

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water *ad libitum*. Nembutal anaesthesia was used for all surgical procedures. Hypophysectomy was performed by the paraphyrangeal approach and checked for completeness at autopsy by binocular examination of the contents of the sella. Hypophysectomized animals were not used until four weeks after operation by which time the adrenal atrophy is virtually complete (Schweizer and Long, 1950). Splenectomized animals were not used until three weeks after operation.

When the blood samples were taken the animals were gently removed from the cage, lightly wrapped in a towel with only the head protruding and allowed to stand on a table while the blood was drawn. Handled in this way the average animal does not struggle at all. Occasionally an animal was found which did not respond quitely to this procedure; such animals were not used for the experiments. Blood was drawn by piercing a superficial ear vein with a number 24 hypodermic needle and waiting until the blood welled-up freely before filling the pipette. Guinea pig blood clots very readily and a freely flowing drop is essential for a reliable sample. The ears were used alternately and a new site chosen for each sample.

Total, differential, and eosinophil counts were all made in the counting chamber on blood diluted 1:20 in Bureau of Standards white cell pipettes with methylene blue-phloxine diluting fluid (Randolph, 1949) freshly mixed from stock solutions for each count. Total white blood cell determinations were estimated by counting the four large corner squares on both sides of a standard Neubauer chamber. The total number of mononuclear cells was counted in the same area using the high power of the microscope. The presence in the guinea pig of pseudoeosinophils (neutrophils) which have a faint pinkish cast makes this differentiation relatively simple. The number of polynuclear leucocytes was determined by subtraction from the total count. The number of eosinophils was estimated by counting the number of cells on all squares on both sides of the Neubauer chamber and all squares on both sides of a Levy counting chamber (depth .2 mm.). Animals with a total eosinophil count below 44/mm.^{3} were not used as the number of cells was considered to small to give a reliable measure of changes. The only exception was in the splenectomized animals in which two having a count of 33/mm.^{3} were included.

The initial count was always made between 9 and 10 A.M. to eliminate the possible effect of diurnal fluctuations (Best and Samter, 1951), and successive counts were made one and three hours later.

Injections of epinephrine (40γ/100 gm. body weight) were given subcutaneously immediately after the blood was drawn for the initial count.

OBSERVATIONS

Resting Level of White Blood Cells. Summarized in Table 1

TABLE 1

Experimental procedure	No. of. Animals	Polynuclear leucocytes ±st. error	Mononuclear cells ±st. error	Eosinophils ±st. error
Normal	12	3230 ± 374.8	5710 ± 372.6	110 ± 24.0
Hypophysectomy	10	3282 ± 688.3	5213 ± 811.7	277 ± 61.1[2]
Splenectomy	9	5590 ± 822.0[1]	7133 ± 952.0	96 ± 38.0

[1] Highly significant difference from normal $p < .01$.
[2] Significant difference from normal $p = .02$.

The initial levels of the polynuclear leucocytes and mononuclear cells were the same in the normal and hypophysectomized guinea pig, but the eosinophil count rose significantly after hypophysectomy. Splenectomy caused a significant rise in the polynuclear leucocytes but no significant alteration in either the mononuclear cell or eosinophil number.

Mild Stress. Summarized in Table 2

Normal animals. The normal guinea pig responded to the procedure for sampling blood by a drop of approximately 30% in both the eosinophil and mononuclear cell count one and three hours after taking the initial sample. That this change was not due to dilution of the blood is shown by the constancy of the polynuclear leucocytes during the same period. This decrease in mononuclear cells and eosinophils, characteristic of the effects of adrenal cortical hormone, indicates that the guinea pig responds to the procedure of sampling blood with a mild stress reaction.

Hypophysectomized animals. Animals hypophysectomized long enough to allow complete atrophy of the adrenal cortex were subjected to the same procedure. As was expected, they showed no fall in the eosinophil count. The mononuclear count, however, decreased consistently though very slightly (16%; $P < .01$) during the first hour but by the third hour alterations were in both directions and not significant ($P = .1$). Thus, only the mild lymphocytopenia during the first hour was independent of the hypophysis. The number of polynuclear leucocytes remained constant throughout.

Splenectomized animals. Animals splenectomized more than 3 weeks were like normal animals with regard to the polynuclear leucocytes (no change) and mononuclear cells (decreased 30%). But unlike the normal, the splenectomized guinea pig showed no alteration in the eosinophil count at the first hour and a smaller (20%) but statistically significant decline by the third hour. Evidently, two mechanisms are involved in the disappearance of eosinophils in the guinea pig subjected to mild stress; the immediate rapid change requires the presence of the spleen but the more prolonged effect involves some other mechanism as it occurred in the splenectomized animal.

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TABLE 2

Experimental procedure	No. of animals	0 hour	1 hour	Dif ± st. error (% change)	0 hour	3 hours	Dif ± st. error (% change)
Polynuclear Leucocytes							
Normal	8	3667	3260	−407 ± 180 (−11.1%)	3667	3888	+221 ± 200 (+6.0%)
Hypx	10	3720*	3539*	−181 ± 273 (−4.8%)	3282	3232	−50 ± 292 (−1.5%)
Splx	9	5591	5472	−119 ± 255 (−2.1%)	5591	5461	−130 ± 575 (−2.3%)
Normal Epin	9	3297	3249	−48 ± 782 (−1.4%)	3297	10847	+7550 ± 839[1] (+235.0%)
Hypx Epin	7	3468	4336	+868 + 554 (+25.0%)	3028	5393	+2365 ± 638[1] (+78.1%)
Splx Epin	8	4650	4768	+118 ± 209 (+2.5%)	4650	16400	+11750 ± 1338[1] (+352.7%)
Mononuclear Cells							
Normal	8	5462	3726	−1736 + 185[1] (−31.7%)	5462	3825	−1637 ± 344[1] (−29.9%)
Hypx	10	5417*	4546*	−871 ± 110[1] (−16.0%)	5213	4373	−840 + 479 (−16.1%)
Splx	9	7133	5047	−2086 ± 550[1] (−29.2%)	7133	5263	−1870 ± 436[1] (−26.2%)
Normal Epin	9	6033	6011	−22 ± 388 (−0.3%)	6033	3942	−2091 ± 384[1] (−34.6%)
Hypx Epin	7	5070	6829	+1759 ± 425[1] (+34.7%)	5407	3961	−1446 ± 602[2] (−26.7%)
Splx Epin	8	7165	6497	−688 ± 528 (−9.6%)	7165	4875	−2290 ± 427[1] (−31.9%)
Eosinophils							
Normal	8	99	70	−29 ± 11[2] (−29.1%)	99	63	−36 ± 14[2] (−36.2%)
Hypx	10	262*	298*	−36 ± 46 (−13.7%)	277	322	+45 ± 46 (+16.2%)
Splx	9	96	97	+1 ± 2 (+1.0%)	96	76	−20 ± 8[2] (−20.8%)
Normal Epin	9	113	161	+48 ± 9[1] (+42.4%)	113	107	−6 ± 18 (−5.3%)
Hypx Epin	7	237	306	+69 ± 39 (+29.1%)	254	369	+115 ± 40[2] (+45.2%)
Splx Epin	8	110	124	+14 ± 8 (+12.7%)	110	118	+8 ± 26 (+7.2%)

* 7 animals.
[1] Highly significant difference $p = .01$.
[2] Significant difference $p < .05$.

Epinephrine. Summarized in Table 2

The effect of subcutaneous injection of epinephrine, which is known to stimulate adrenal cortical activity (Recant, Hume, Forsham and Thorn, 1950) was studied in the three groups of animals.

Normal animals. The normal animal injected with epinephrine immediately after the initial sampling of blood did not respond with the rapid decrease in mononuclear cells and eosinophils characteristic of the guinea pig in mild stress. At the end of the first hour the mononuclear cells were unchanged and the eosinophils had increased (42%) but by the third hour the mononuclear cells had fallen to 35% of their initial value and the eosinophils were back to their initial level. A comparison, however, of the count taken three hours after injection with the first hour count showed a decline of 33% in mononuclear cells and of 33.5% in eosinophils ($P = .02$). These data suggest an early mobilization of mononuclear cells and eosinophils sufficient to mask any decrease the first hour. This early change was not a general mobilization of all cell types as the polynuclear leucocytes were unaltered at the end of the first hour. However, 3 hours after the injection the polynuclear leucocytes increased greatly (235%).

Hypophysectomized animals. During the first hour after injection of epinephrine into the hypophysectomized guinea pig there was a marked rise in mononuclear cells but by the third hour there was a significant decrease (26%) from the initial count. Moreover, the count at the third hour compared with that at the end of the first hour showed a fall of 41.7%. Although the decline in number of mononuclear cells after the injection of epinephrine occurred in the absence of the hypophysis this was not true of the eosinophils. This cell type showed no significant change at the first hour and increased (45%) by the third hour after injection. The polynuclear leucocytes were not significantly altered at the end of the first hour and increased (78%) by the third hour.

Splenectomized animals. Changes in the polynuclear leucocytes and mononuclear cells of the injected splenectomized guinea pig paralleled those in the injected normal animal. The mononuclear cells were unchanged the first hour and fell 32% by the third hour after injection; the polynuclear leucocytes also showed no alteration during the first hour but increased markedly (352%) by the third hour. Although alterations in the polynuclear leucocytes and mononuclear cells after epinephrine were independent of the spleen, the rise in eosinophils at the first hour in the injected normal guinea pig was absent in the splenectomized animal. The delayed mobilization of polynuclear leucocytes by epinephrine is independent of the spleen and presumably arises from the bone marrow, but the more rapid mobilization of eosinophils one hour after injection requires the presence of the spleen.

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DISCUSSION

In the preliminary experiments designed to standardize the method for obtaining blood from the ear vein of the unanaesthetized guinea pig it was evident that the procedure itself acted as a mild stress in this species as it does in the mouse (Speirs and Meyer, 1949). Speirs and Meyer (1949) also report a moderate eosinopenia after handling in mice hypophysectomized 3–14 days but, as reported here, guinea pigs hypophysectomized long enough to allow complete atrophy of the adrenal gland (over 4 weeks) did not show any decrease in eosinophils after sampling blood. The characteristic lymphopenia 3 hours after mild stress was also lacking in the absence of the hypophysis. Colfer, de Grott and Harris (1950) also found no lymphopenia after emotional stress in the hypophysectomized rabbit. There was, however, a very slight (-16%) but consistent decrease in the mononuclear cells in the hypophysectomized guinea pig one hour after the first sample was drawn. Hungerford (1949) has reported a small decrease in lymphocytes in the hypophysectomized (but not in the adrenalectomized) rat which he attributes to a direct effect of epinephrine on the adrenal cortex and in the experiments reported here epinephrine also caused a decrease in mononuclear cells 3 hours after administration to the hypophysectomized guinea pig. The very small fall in mononuclear cells during the first hour in the hypophysectomized guinea pig subjected to mild stress may be due to endogenous epinephrine acting directly on the adrenal gland. Hungerford (1949), however, found no such effect of endogenous epinephrine in the hypophysectomized rat.

The role of the spleen in the alteration of the white blood cell picture is somewhat ambiguous. Spain and Thalhimer (1951) attribute the eosinopenia following administration of cortisone to the mouse to a migration into spleen, but Fruhman and Gordon (1952) have found eosinopenia in the blood in the rat consistently correlated with an eosinopenia in the spleen. Eosinopenia is reported by Speirs and Meyer (1949) in the splenectomized mouse after mild stress and by Dury (1950) and McDermott, Fry, Brobeck and Long (1950) in the rat after epinephrine injection. Padawer and Gordon (1952) attribute the eosinopenia after cortisone and epinephrine in the rat to degenerative changes in the circulating eosinophils. The data reported in this paper indicate that more than one mechanism is involved in the eosinopenia following mild stress in the guinea pig. Eosinophils remain constant during the first hour in the splenectomized animal but decrease after 3 hours. Whether the initial fall, which requires the presence of the spleen, is due to sequestration in that organ can only be answered conclusively by direct studies of the type done by Fruhman and Gordon (1952). The later changes which are independent of the spleen probably occur by the mechanism of Padawer and Gordon (1952).

The initial rise in eosinophils induced one hour after administration of epinephrine (McDermott *et al.*, 1950; Padawer and Gordon, 1952) is absent in the splenectomized rat (McDermott *et al.*, 1950). This is confirmed for the guinea pig in this report.

Dury (1950) found an absence of the polynuclear leucocyte response to epinephrine in the splenectomized rat, but Stein, Martin and Falk (1951) obtained a neutrophilia in the splenectomized mouse and Bierman, Kelly, King and Petrakis (1951) found an increase in leucocytes in splenectomized patients. The data in this paper are in agreement with the latter reports. In fact, the increase in polynuclear leucocytes after epinephrine is even greater in the splenectomized than in the normal guinea pig.

The most atypical finding was the failure to obtain a clear-cut eosinopenia 3 hours after the injection of epinephrine such as is consistently found in other species (rat, mouse, man). Samter, Erickson and Kofoed (1952) also found the guinea pig sluggish in its reaction to ACTH. Since the normal and splenectomized guinea pig showed a characteristic eosinopenia in response to mild stress the eosinophils of the guinea pig cannot be refractory to the effect of cortical hormone. It seems probable that the decrease in eosinophils in animals injected with epinephrine and ACTH is being masked by a simultaneous outpouring of these cells. The eosinopenia at the first hour is masked by the mobilization from the spleen discussed above. By the third hour after epinephrine there is a great mobilization of polynuclear leucocytes, presumably, from the bone marrow (since it also occurs in the splenectomized guinea pig). Samter *et al.* (1952) remark that the injection of ACTH also caused a large increase in polynuclear leucocytes. If, as seems likely, this mobilization of leucocytes from the bone marrow is accompanied by eosinophils it explains the masking of the characteristic eosinopenia. The rats in our own colony have a later time (6–8 hrs.) of optimal response to epinephrine than the Sprague Dawley stain (4–6 hrs.), (Gordon, personal communication). The possibility that the guinea pig will show a similarly delayed response is being explored.

SUMMARY

The procedures involved in sampling blood from the ear vein in the unanaesthetized guinea pig induce a mild stress reaction. Within one hour after drawing a blood sample the number of mononuclear cells and eosinophils have decreased approximately 30% and this fall is sustained through the third hour. The number of polynuclear leucocytes is unaltered during this interval. The decline in eosinophils is dependent upon the presence of the hypophysis. The initial decrease in eosinophils requires the presence of the spleen but the later disappearance occurs in the splenectomized animal.

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Epinephrine administered subcutaneously causes a lymphopenia three hours after injection which is independent of the hypophysis and a marked polynuclear leucocytosis which is independent of the spleen. The characteristic eosinopenia anticipated 3 hours after epinephrine administration does not occur. This atypical response is discussed.

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THE ASSAY OF ESTROGENS IN THE CHICK BY ORAL ADMINISTRATION[1]

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THIS communication is concerned with the bioassay of estrogens in chicks when the material is administered orally and with the relative activities of various estrogens by the oral route.

ANIMALS, MATERIALS, AND METHODS

Various preparations of estrogen sulfates were supplied by Endo Products Inc.; the stilbestrol was supplied by Winthrop-Stearns Inc.; and methoxy bisdehydrodoisynolic acid (MDDA) and estradiol dipropionate was supplied by Ciba Pharmaceutical Products Inc. The *Premarin* (Ayerst, McKenna and Harrison) was purchased on the open market. Estynyl estradiol was supplied by the Schering Corporation.

The experimental design of Bliss (1944) was employed for statistical calculations of potency ratio. This consisted essentially in setting up four groups of animals, two groups on the standard and two groups on the unknown. The concentrations of the high and low doses were determined by the following relationship:

$$\frac{\text{High Dose Standard}}{\text{Low Dose Standard}} = \frac{\text{High Dose Unknown}}{\text{Low Dose Unknown}} = \frac{2}{1} \text{ or } \frac{4}{1}$$

Using this experimental design, the combined slope (b), the significance of the differences of the slopes of unknown and standard (t), the potency ratio, and the standard error of the potency ratio were calculated.

Sex-linked pullets obtained from Kerr Chickeries, Frenchtown, New Jersey, were used 2 to 3 days after hatching. They were placed on a diet of chick starting mash to which the estrogen was added as specified under the individual experiments. After 13 days on this diet the chicks were autopsied, at which time the body and oviduct weights were determined. The oviduct ratio is defined as the milligrams of oviduct per 100 gm. of body weight.

EXPERIMENTAL

Tables 1 and 2 list the oviduct responses to a variety of estrogens administered in the food. Table 1 is concerned with naturally occurring

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TABLE 1. CHICK OVIDUCT RESPONSES TO NATURALLY OCCURRING ESTROGENS AND DERIVATIVES

(Chicks on Diet 13 days, starting 2–3 days after hatching)

Preparation	Dosage estrogen sulfate mg. per kg. food	Number of chicks	Mean oviduct Ratio ±S.E.*
0	0	32	16 ± 0.5
0	0	24	16 ± 0.5
0	0	37	14 ± 0.5
Sodium Estrone Sulfate (Pure)	40	30	31 ± 4.0
	80	30	115 ± 11.0
	80	16	117 ± 17.0
	160	16	466 ± 90.0
Sodium Estrone Sulfate (Crude)	40	30	39 ± 3.8
	80	27	142 ± 19.0
Commercial Preparation (Premarin)	40	97	63 ± 8.0
	80	92	192 ± 15.0
Sodium Estradiol Sulfate	40	14	28 ± 4.0
	80	29	78 ± 13.0
	160	14	470 ± 129.0
Estrone	20	25	24 ± 2.0
	40	26	40 ± 3.0
	80	24	149 ± 24.0
	160	27	278 ± 21.0
Estradiol Dipropionate	80	23	77 ± 6.0
	160	24	202 ± 36.0
Estynyl Estradiol	2.5	33	23 ± 1.0
	5.0	30	26 ± 3.4
	10.0	33	28 ± 1.4
	20.0	29	88 ± 11.0

* S.E. = Standard Error

estrogens while Table 2 is concerned with two synthetic estrogens.

Assay using the Bliss design

The evaluation of the assay using the Bliss design is illustrated by Table 3. Here a total of 44 chicks were employed (22 on the standard and 22 on the unknown). The theoretical potency was 100% since the commercial preparation *Premarin* was assayed against itself. The mean standard error of the potency ratio was found to be ±12%. The mean value for λ, the index of precision, was found to be 0.178. The slopes of the unknown and standards were not significantly different.

In Table 4 three assays are indicated in which two samples of sodium

TABLE 2. CHICK OVIDUCT RESPONSE TO SYNTHETIC ESTROGENS

Estrogen	Dose of estrogen mg. per kg. food	No. of chicks	Mean oviduct ratio ±S.E.
0	0	32	16 ± 0.5
MDDA	5	30	17 ± 1.0
	10	32	24 ± 1.5
	20	40	26 ± 1.1
	40	18	42 ± 3.0
	80	20	88 ±16.0
	160	19	523 ±88.0
Stilbestrol	5	44	20 ± 0.6
	10	78	22 ± 0.6
	20	79	33 ± 1.0
	40	73	67 ± 3.0
	80	38	205 ±43.0
	160	34	480 ±45.0

estrone sulfate were compared. A highly purified sample was considered as the standard and an impure sample of sodium estrone sulfate was the unknown. Two doses of each preparation at 40 mg. and 80 mg. were employed and the data analyzed by the method of Bliss (1944). The slopes did not differ significantly and the potency ratios in three different trials did not differ significantly from 100%, indicating that on an estrogenic activity basis no difference between the two preparations was demonstrable. When a total of 48 animals was used the standard error of the potency ratio was 9%, with 56 animals it was 13%, and with 104 animals it was 8%.

Table 5 is a summary of the comparative activities of various estrogens. The comparative activities of the seven estrogens were compared amongst themselves by choosing pairs of compounds whose log dose-response curves gave similar slopes. That is, slopes of a given standard and unknown were not significantly different. Stilbestrol, estrone, and sodium estrone sulfate

TABLE 3. THE ASSAY OF ESTROGEN SULFATES (*Premarin*)

$$\frac{\text{High Dose Unknown}}{\text{Lose Dose Unknown}} = \frac{\text{High Dose Standard}}{\text{Low Dose Standard}} = \frac{\text{80 mg./Kilo}}{\text{40 mg./Kilo}}$$

Unknown = Standard

N	Slope b	Index of precision λ	t	Potency ratio % ±S.E.%
11	445	0.112	1.950	117 ± 9
11	692	0.206	0.082	102 ±14
11	363	0.198	0.276	103 ±14
11	385	0.246	0.906	123 ±22
11	633	0.137	1.752	118 ±11
11	568	0.173	1.680	118 ±15

TABLE 4. THE RELATIVE ACTIVITY OF PURE AND CRUDE SODIUM ESTRONE SULFATE

$$\frac{\text{High Dose (Impure)}}{\text{Low Dose (Impure)}} = \frac{\text{High Dose (Pure)}}{\text{Low Dose (Pure)}} = \frac{\text{80 mg./Kilo}}{\text{40 mg./Kilo}}$$

N	Slope b	λ	Significancy of Difference between slopes t	Potency ratio S.E. ± %
12	268	0.131	1.021	101 ± 9
14	341	0.206	1.262	119 ± 15
26	306	0.188	0.684	111 ± 9

were chosen as the standards in the various assays. The error range was reasonably low. The highest error range at P = 0.95 being −18 to +22%. For these calculations the methods of Irwin (1937) and Bulbring (1938) were employed.

DISCUSSION

The method described in this communication affords a convenient objective means to assay estrogenic compounds. The method is simple, inexpensive, and lends itself to economy of space in keeping the animals and time of personnel. The accuracy has been found to be acceptable. Thus,

TABLE 5. RELATIVE ACTIVITY OF ESTROGENS ADMINISTERED ORALLY

Standard	Unknown	Dose range mg./kilo	No. of chicks	Potency ratio %	Error range % P = 0.95
Stilbestrol		80; 160	72		
	Sodium Estrone Sulfate	80; 160	62	87	−12; +14
Stilbestrol		20; 40	152		
	Estynyl Estradiol	10; 20	62	226	− 9; +10
Stilbestrol		40; 80	111		
	Estrone	80; 160	51	74	−14; +17
Estrone		40; 80; 160	77		
	Estradiol Dipropionate	80; 160	47	64	−18; +22
Sodium Estrone Sulfate		80; 160	62		
	MDDA	80; 160	39	100	−12; +13
Sodium Estrone Sulfate		40; 80; 160	60		
	Sodium Estradiol Sulfate	40; 80; 160	59	100	−16; +19

using a total of 44 chicks ($\frac{1}{2}$ on the standard and $\frac{1}{2}$ on the unknown) resulted in a mean standard error of the potency ratio of 12.6% in seven trials. The mean value of λ, a measure of the precision of the assay, was 0.178. The slopes of unknown and standard did not differ significantly.

TABLE 6. RELATIVE ORAL ACTIVITY OF VARIOUS ESTROGENS IN CHICKS
(Estrogenic Potency of Estrone = 100%)

Estrogen	Relative potency %
Estynyl Estradiol[2]	300
Sodium Estrone Sulfate[1]	164
Sodium Estradiol Sulfate[2]	163
Stilbestrol	137
MDDA	119
Estrone	100
Estradiol Dipropionate[2]	90

[1] Calculated as Estrone.
[2] Calculated as Estradiol.

The method was used to evaluate the relative potencies of seven representative estrogens. These findings are summarized in Table 6. The potency of estrone was set at 100% and the other estrogens compared to the activity of this estrogen. The relative activities in descending order were estynyl estradiol (300%), sodium estrone sulfate (164%, calculated as estrone), sodium estradiol sulfate (163%, calculated as estradiol), stilbestrol (137%), MDDA (119%), and estradiol dipropionate (90%, calculated as estradiol).

SUMMARY

A quantitative method for the oral bioassay of estrogens is presented. The end point is the oviduct weight response of pullets. The method is convenient, inexpensive, and reproducible. When a total of 44 chicks was employed (22 on standard and 22 on the unknown) the potency ratio was determined with a mean standard error of $\pm 12.6\%$.

The oral relative potencies of seven estrogens is presented. Considering estrone as having a potency of 100%, the following potencies were determined: estynyl estradiol, 300%; sodium estrone sulfate, 164% (calculated as estrone); sodium estradiol sulfate, 163% (calculated as estradiol); stilbestrol, 137%, MDDA, 119%; and estradiol dipropionate, 90% (calculated as estradiol).

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EFFECTS OF TESTOSTERONE AND OTHER STEROIDS ON INCORPORATION OF GLYCINE-1-C^{14} INTO PROTEIN BY RAT AND HUMAN LIVER SLICES[1]

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THE specific role of many hormones in tissue metabolism remains obscure. The simple addition of pure hormones to *in vitro* systems might be expected to offer some useful information with respect to the mechanism of action of such hormones. Since some steroid hormones are known to influence protein metabolism in the intact animal, such hormones were added directly to liver slices and their effects upon the incorporation of radioactive glycine into protein were observed. This report presents data which demonstrate a significant depression of incorporation with certain steroids and that the depression is the same for rat and human liver tissue.

EXPERIMENTAL

Liver tissue was sliced and incubated, the protein precipitated, prepared for counting, counted in a gas-flow counter, and corrected for self-absorption as previously described (Grossman and Hauschildt, 1952). Steroids[3] dissolved in ether were added to the incubation flasks and the ether was evaporated by gentle heat before adding buffer and tissue. 30 to 300 μg. of steroid were added to approximately 100 mg. of tissue in each flask, together with 0.05 mg. of glycine-1-C^{14} (0.5 microcurie/millimole[4] and 140,000 c/m in our counter) in 2 cc. of Krebs Henseleit buffer. Rats were killed by a blow on the back of the head, the liver was removed, placed into chilled buffer, and sliced by hand.

All experiments were performed with duplicate flasks for each set of

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[3] Crystalline steroids supplied by Ciba Pharmaceutical Products, Summit. N. J.

[4] Purchased from Tracerlab, Inc., Boston, Mass.

conditions, and the results are expressed as means for each set of duplicate pairs.

RESULTS

Table 1 presents the effects of added hormones and cholesterol upon glycine incorporation. Since the incorporation into protein is variable from animal to animal each set of experimental conditions was paired with a control from the same animal. The effect of testosterone was uniformly depressing, and the difference from the controls is statistically significant ($p < .001$). Cholesterol did not have a uniform depressing effect, and the difference from the control was not significant. Desoxycorticosterone and estradiol also inhibited the incorporation, whereas cortisone did not produce as uniform a depression. The addition of diphosphopyridine nucleotide (DPN) did not reverse the depression caused by testosterone in four

TABLE 1. EFFECT OF ADDED TESTOSTERONE, CHOLESTEROL, DESOXYCORTICOSTERONE, ESTRADIOL, CORTISONE, AND TESTOSTERONE + DPN ON INCORPORATION OF GLYCINE-I-C^{14} INTO RAT LIVER SLICES

Each control represents data from a single animal selected at random as to sex. Buffer, Krebs-Henseleit containing 0.05 mg. glycine-I-C^{14} (140,000 Cts/min/flask), approx. 100 mg. liver slices (wet weight). Incubation 4 hrs. Temp. 37°. Gas phase 95% O_2–5% CO_2

	Control	Testosterone			Cholesterol			Desoxycorti-costerone			Estradiol			Cortisone			Testosterone +2.3 mg. DPN	
	Cts/min. /mg. protein	Cts /min. /mg.	mg. added	% change	Cts /min. /mg.	mg. added	% change	Cts /min. /mg.	mg. added	% change	Cts /min. /mg.	mg. added	% change	Cts /min. /mg.	mg. added	% change	Cts /min. /mg.	% change
	670	570	.2	−15	870	.3	+30							500	.3	−43		
	410	210	.2	−49	250	.3	−39											
	560	140	.2	−75	1140	.2	+104											
		500	.1	−11														
		540	.05	− 4														
	890	470	.2	−47	630	.2	−29	320	.2	−64	350	.2	−61	710	.2	−20		
	420	250	.1	−40	250	.1	−40							250	.1	−40	500	+19
	840	670	.1	−20	830	.1	−1							1020	.1	+21	550	−35
	420	170	.2	−60				90	.3	−79				80	.3	−81		
		230	.1	−45														
		270	.05	−36														
	240	150	.3	−38				40	.3	−83				430	.3	+79		
	660	330	.1	−50										370	.1	−44	360	−45
	700	310	.1	−56				440	.1	−37							240	−66
	480	310	.1	−35				480	.1	0							200	−58
	560	230	.1	−59				560	.1	0	270	.1	−52	600	.1	+ 7		
	220				220	.1	0				230	.1	+ 5					
	520				300	.1	−42	290	.1	−44	220	.1	−58	280	.1	−42		
	130				150	.3	+15											
	440				300	.3	−35											
	440				320	.3	−27											
	370				210	.3	−43											
	310				210	.3	−32											
	1150										870	.1	−24					
	970							880	.1	− 9	560	.1	−42	180	.1	− 83		
Zero time ± SE	11± 3																	
Mean				−40			−11			−40			−39			−26		−37
Mean diff.* ± SE				209± 35			42± 50			227± 75			278± 87			109± 66		250± 123
p values†				<.001			>.4			<.05			<.05			>.10		<.10

* Mean of the differences: control minus testosterone for each experimental pair, and these differences are averaged. The differences of the other experimental pairs were similarly averaged.

† p Values taken from Student's table for t (Fisher and Yates 1947).

out of five experiments. The results with human liver slices and testosterone are presented in Table 2. All the human liver specimens were histologically normal.

TABLE 2. EFFECTS OF ADDED TESTOSTERONE UPON THE INCORPORATION OF GLYCINE-1-C^{14} INTO PROTEIN BY HUMAN LIVER SLICES

Buffer, Krebs-Henseleit containing 0.05 mg. glycine-1-C^{14} (140,000 Cts/min/flask), approx. 100 mg. liver slices (wet weight). Incubation 4 hrs. Temp. 37°. Gas phase 95% O_2—5% CO_2

			Control	Testosterone		
Patient	Age	Sex	Cts/min/mg. Protein	Cts/min /mg.	mg. added	% change
I.T.	71	M	210	140	.3	−33
W.H.	77	M	130	200	.06	+54
				160	.03	+23
A.K.	66	F	610	450	.03	−45
				210	.01	−66
E.S.	23	F	250	140	.3	−44
G.F.	76	F	690	380	.3	−45
D.H.	35	F	750	390	.3	−48
F.O.	69	M	350	130	.3	−63
H.C.	79	M	740	200	.3	−73
G.K.	77	F	740	420	.06	−43
J.M.	66	M	580	270	.06	−54
L.S.	72	M	710	970	.03	+37
A.A.	62	F	470			
Zero Time Mean ±SE			12 ±5			
Mean						−32
Mean Diff.* ±SE				242 ±68		
p Value†				< .01		

* Mean of the differences: control minus testosterone for each experimental pair, and these differences are averaged.

† p values taken from Student's table for t (Fisher and Yates 1947).

Variations in the amount of testosterone present yielded no consistent difference in the degree of inhibition. Statistical evaluation of the inhibition in the presence of 100 and 200 μg. revealed that the results were the same.

Samples of labeled liver protein were dissolved in alkaline urea and reprecipitated with trichloracetic acid with no essential change in specific activity.

DISCUSSION

Although there is no definitive proof that glycine incorporation into protein by liver tissue *in vitro* represents true protein synthesis, considerable evidence does exist which demonstrates that the incorporation is an enzyme mediated process requiring oxidative phosphorylation and that labeled glutathione as well as labeled protein has been obtained with such systems (Greenberg *et al.*, 1948; Zamecnik *et al.*, 1948; Borsook *et al.*,

1950; Peters and Anfinsen, 1950; Simpson and Tarver, 1950; Grossman and Hauschildt, 1952; Siekevitz, 1952; Snoke and Bloch, 1952; and Krahl, 1953). In addition, Anfinsen and Steinberg (1952) and Anfinsen and Flavin (1953) have obtained labeled peptide fragments by enzymatic digestion of labeled protein which had been from an *in vitro* system. The recent report of Brunish and Luck (1952) suggests that caution should be exercised in interpreting low levels of incorporation in homogenate and particulate systems. No similar data are available suggesting peculiar reactions with liver slices, and it is probable that the slice system with its fundamentally intact cells will be more apt to reproduce *in vivo* biochemical reactions than will homogenate and particulate systems. Furthermore, solution and reprecipitation of the labeled protein did not alter its specific activity.

It is now well known that the radioactive protein obtained after incubation with glycine-1-C^{14} yields labeled serine as well as labeled glycine upon hydrolysis (Winnick *et al.*, 1948, and Zamecnik and Frantz, 1949). This results from the ready conversion of glycine to serine in the liver slice system. We recently confirmed this using labeled protein obtained by incubating human liver slices with glycine-1-C^{14} (Grossman *et al.*, 1953).

Since testosterone is considered to be a protein anabolic hormone, one might expect an increased incorporation of glycine into protein under its influence. The fact that the opposite result was found is not surprising. Samuels (1950) has shown that liver tissue destroys testosterone and that oxygen and DPN are required for the optimum conversion to a 17-ketosteroid. Some of the known requirements for glycine incorporation into protein are oxygen, Mg++, and adenosinetriphosphate (Winnick, 1950; Grossman and Hauschildt, 1952; Siekevitz, 1952). It is quite possible that the available energy and coenzymes were being utilized for the two reactions at one time, resulting in some inhibition of incorporation. DPN, however, which has been shown to accelerate the destruction of testosterone (Samuels, 1950), did not overcome the inhibition of this hormone to any degree. Hayano and Dorfman (1951) have shown that desoxycorticosterone and other steroid hormones depress some liver enzyme systems and at the same time depress oxygen consumption. Kochakian (1951) has also shown that testosterone will depress the oxygen consumption of liver slices, although the amount of testosterone used was far in excess of physiological concentrations. In these experiments the amounts of steroid used were considered to be within physiological ranges and of the order of 0.5 millimolar concentration. Kalman (1953) demonstrated inhibition of both succinoxidase and malic dehydrogenase of rat liver by testosterone and suggested some type of adsorption phenomenon involving enzyme and steroid. Recently Kit and Guzman Barron (1953) reported that adrenal steroids inhibited the incorporation of C^{14} glycine into lymphatic cell pro-

tein without much effect on respiration, and suggested a specific inhibition of protein synthesis by an unknown mechanism. This may be the explanation for our findings, but the fairly uniform depression of incorporation with various steroids suggests the possibility of a non-specific effect.

The sex of the animal or patient used seemed to bear no relation to the results obtained with either testosterone or estradiol.

SUMMARY

The addition of testosterone, estradiol and desoxycorticosterone to rat liver slices depressed the incorporation of glycine-1-C^{14} into protein. Cortisone caused some depression but was less effective, whereas cholesterol produced no significant depression.

The same effects of testosterone were observed with liver slices from human biopsy material.

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GLUCOSE STUDIES IN CROCODILIA

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IN A continuation of a study of the biochemistry of Crocodilia (Coulson, Hernandez and Brazda, 1950; Coulson, Hernandez and Dessauer, 1950; and Hernandez and Coulson, 1952) efforts have been directed toward an understanding of those aspects of glucose utilization which can be observed in the intact animal. This communication deals with the fate of injected glucose in the alligator and caiman and with various factors which influence its storage and metabolism. It is hoped that this paper may help to throw new light on the interpretation of "glucose tolerance" curves in the reptiles.

EXPERIMENTAL

The alligators (*A. mississippiensis*), were obtained from the swamps of Louisiana and those used in this study were from 8 inches to 5 feet in length and weighed from 70 gm. to 8 kg. The caimans (*C. latirostris*), weighing 100 gm. to 1 kg., were obtained from a dealer who imported them from South America. Blood sugars were determined by the Folin-Wu method and glycogens by the method of Good, Kramer and Somogyi (1933). Thiocyanate space was determined by a modification of the method of Crandall and Anderson (1934). All of the animals used in the adrenalin, insulin, glucose and glycogen studies were deprived of food for 3 days before use. It was necessary to feed the alligators used for the study of the effects of ACTH and cortisone at regular intervals during these lengthy investigations. When the colony was not in use for experiments it was maintained on a diet of rat meat fed twice weekly.

RESULTS

Glucose Tolerance

Preliminary experiments established the fact that the magnitude of the blood glucose rise was proportional to the dose. Each of 78 alligators and 18 caimans were given a single intraperitoneal injection of one gram of glucose regardless of the size of the animal. Whereas the blood glucose content increased to more than 2000 mg.% in the smallest animals (70 gm.), the glucose rise in the largest (8 kg.) was imperceptible. Although it

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was possible to show that the absolute renal threshold was about 800 mg.%, the rate of excretion of glucose when the blood level was as high as 1200 mg.% was so slow that renal loss was almost negligible. By plotting the inverse of the body weights of the 96 animals against the maximum glucose rise a straight line resulted. Since the height of the rise seemed to be controlled solely by the size of the animal the influence of hormones on this "tolerance" to glucose seemed to be of minor importance. The intact alligator resembles an eviscerated mammal in its reaction to exogenous glucose.

To avoid renal loss the experiment was repeated by giving each animal 3.8 gm./kg. which was the amount calculated to keep the maximum blood level just below the renal threshold at about 800 mg.%. The highest blood glucose value obtained from each of the various sized alligators and caimans was used to determine the volume of fluid in which the glucose was distributed. This apparent "glucose space" is presented in Figure 1. Since both alligators and caimans reacted identically in these experiments they were used interchangeably.

If one assumes a straight line from the points in Figure 1 it would appear that the glucose is diluted by an amount of fluid equal to about 50% of the body weight. When animals of approximately one kg. body weight were analyzed separately from those weighing 70–140 gm. the average fluid volume used in dilution seemed to be of the order of 47% in the large animals and 55% in the small ones. It was considered possible that these high figures for glucose space represent the approximate percent of extracellular fluid.

In order to compare thiocyanate space with this glucose space fifty alligators previously used in the glucose experiments and weighing from 90 gm. to 5 kg. were each injected intracardially with 20 mg. of NaCNS per kg. of body weight. The peak blood level of NaCNS was determined and the theoretical extracellular fluid volume was computed. The smaller animals had an average thiocyanate space of 51.4% of the body weight and the larger ones 41.1%. If a correction is made for the glucose which may have been disposed of by the animals at 28°C. temperature in 6 hours time (Hernandez and Coulson, 1952), the glucose space is 50% in the small animals and 42.5% in the large ones. Although this is in close agreement with the space determined by the thiocyanate experiments it may be unsafe to conclude that no difference exists. By the use of Carbon-14 labelled glucose Wick *et al.* (1950) were able to show that thiocyanate space in eviscerated rabbits is almost identical with glucose space. The volume of this space in intact small alligators is considerably larger than that of the mammals so far investigated.

Effect of temperature on glucose tolerance. Figure 2 presents the results of

the effect of temperature on the duration of the "glucose tolerance" curve. The two curves were obtained from the same group of ten animals and in both experiments each alligator received 3.8 gm. of glucose/kg. At 28°C. the average time required to dispose of the glucose was about 72 hours; at 16°C. over 180 hours were required. The injection of 3.8 gm. glucose/kg. did not cause the deposition of an appreciable amount of either hepatic or extra hepatic glycogen in another group of four animals analyzed (Table 1). If we make the not necessarily valid assumption that the rate of disappearance of the blood glucose is determined primarily by its metabolism and not by its conversion to glycogen it is possible to calculate the approximate metabolic rate. The time required for the glucose to disappear in each of the 10 animals in Figure 2 was noted and the oxygen

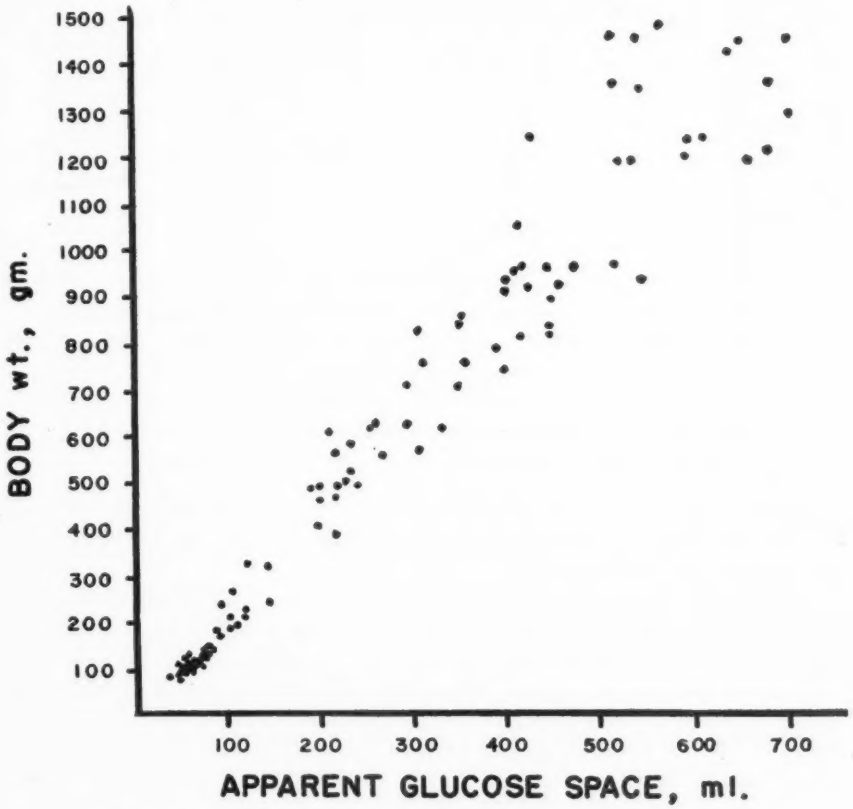

FIG. 1. The apparent "glucose space" in alligators and caimans of different sizes. Each dot represents the value for 1 animal.

necessary to burn this glucose was computed. The group at 28° would require an average of 1.27 cc. of O_2/gm./day; the same group would require 0.33 cc. O_2/gm./day at 16°C. Since the calculated rate of oxygen utilization by more than 50 alligators at 28° is close to that previously reported for oxygen consumption by actual metabolism tests on alligators and caimans of comparable size (Hernandez and Coulson, 1952), it seems probable that the rate of glucose disposal is proportional to the metabolic rate. Small

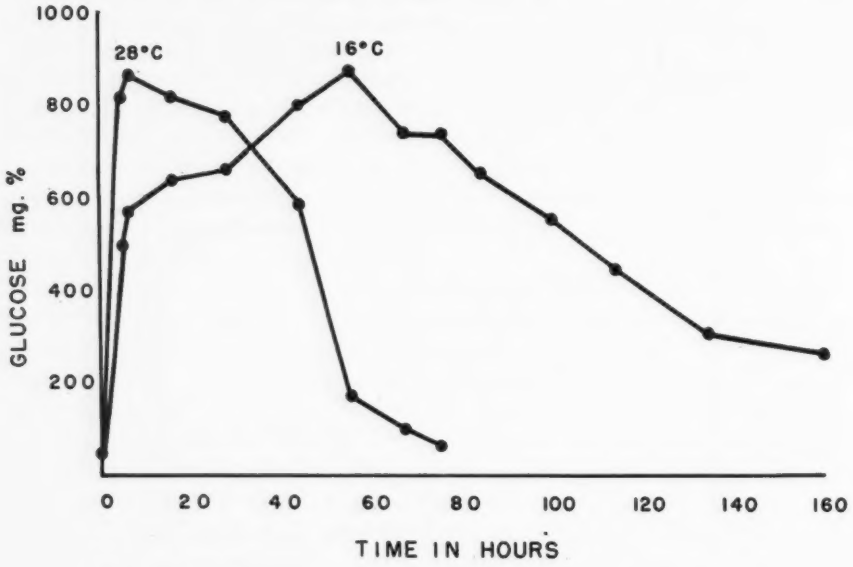

FIG. 2. Effect of temperature on the "glucose tolerance" curve. Each alligator received 3.8 gm. glucose/kg. Average of 10 animals.

alligators disposed of glucose proportionally faster than large ones which also demonstrates the probable relationship between the rate of glucose disappearance and metabolic rate.

The effect of insulin on blood glucose. The failure of alligators to store or otherwise remove much of the glucose for periods of many hours following injection could be due either to a very low production of insulin or to a very low sensitivity to insulin. Sixty alligators were injected intramuscularly with insulin in doses which varied from 0.001 U./gm. to 1 U./gm. In all cases 0.01 U. of insulin*/gm. of body weight produced a measurable response and 0.001 U./gm. usually produced a slight response. From this it may be inferred that although the alligators are much less sensitive to insu-

* Insulin, Iletin, Eli Lilly.

TABLE 1. THE EFFECT OF GLUCOSE, INSULIN, AND ADRENALIN ON THE AMOUNT AND DISTRIBUTION OF GLYCOGEN

Group	Liver % body wt	Liver glycogen, %	Liver glycogen as % body wt	Extrahepatic glycogen, %	Total glycogen, %	No. alligators
Control	2.56* (1.99–3.10)†	7.50 (5.53–10.62)	0.19 (0.14–0.21)	0.32 (0.17–0.37)	0.49 (0.31–0.59)	7
Glucose 3.8 gm./kg.	2.32 (1.61–2.55)	7.39 (4.43–11.14)	0.17 (0.08–0.33)	0.33 (0.16–0.53)	0.50 (0.27–0.73)	4
Glucose 11.3 gm./kg.	2.96 (1.86–3.54)	12.18 (10.44–15.00)	0.36 (0.20–0.48)	0.21 (0.14–0.28)	0.57 (0.36–0.73)	4
Glucose 11.3 gm. /kg. +insulin 0.1 U./gm.	3.01 (2.23–3.64)	11.14 (10.00–12.32)	0.34 (0.22–0.67)	0.23 (0.18–0.31)	0.56 (0.53–0.57)	4
Glucose 22.0 gm./kg.	2.92 (2.74–3.09)	13.58 (12.65–14 42)	0.40 (0.39–0.40)	0.58 (0.49–0.68)	0.96 (0.87–1.05)	2
Glucose 22.0 gm. /kg. +insulin 1.0 U./gm.	3.42 (2.61–4.70)	15.38 (13.38–17.24)	0.53 (0.35–0.79)	0.68 (0.51–0.92)	1.19 (0.86–1.47)	4
Adrenalin 2 cc./kg.	2.11 (1.70–2.45)	3.05 (1.58–4.20)	0.06 (0.30–0.09)	0.18 (0.14–0.23)	0.23 (0.22–0.25)	5

* Mean.
† Range.

lin than mammals they are much more sensitive than the chameleons, *A. carolinensis* (Dessauer, 1953).

The administration of insulin in amounts greater than 0.1 U./gm. produces a state of "shock" within a few minutes. This condition which occurs during the period of hyperglycemia is characterized by nervous twitches, teeth chattering and muscular incoordination. The alligator gradually improves and in 4 or 5 hours appears to be normal. A second state of shock appears about 36 hours after insulin. This disorder is accompanied with and is probably due to hypoglycemia and is invariably fatal unless the animal is treated with frequent and massive injections of glucose.

The hypoglycemic effect of injected insulin on blood glucose is not a rapid one. Hypoglycemia was usually not manifest for several hours and when doses higher than 0.01 U./gm. were injected an initial hyperglycemia of considerable duration was noted. Figure 3 presents the results of one of several experiments on the effects of the injection of 1.0 U. and 0.01 U. of insulin/gm. into alligators at 28°C. By keeping the alligators alive through frequent injections of glucose it was possible to demonstrate that the hypoglycemic effect of 1 U. of insulin/gm. lasted about 20 days. The effect of 0.1 U. lasted about 10 days and of 0.01 U. about 5 days.

The very considerable hyperglycemic rise immediately following the

injection of commercial "insulin" usually lasts from 10 to 20 hours. It was possible to determine that this rise was not due to insulin since it was still apparent when the insulin was inactivated by incubation with 0.08 N KOH at 36°C. for 3 hours according to the method of Sutherland and Cori (1948), (Fig. 3). This hyperglycemia which follows the administration of amorphous commercial insulin is somewhat more marked and very much more prolonged than that observed for mammals; in a few cases the blood glucose increased by as much as 250 mg.%. It is probable that this rise is due to increased glycogenolysis in the alligator similar to that reported for the rabbit (Drury, 1935; Wick *et al.*, 1951).

The effect of insulin and glucose on body glycogen. Twenty-five small alligators were available for the determination of the effect of glucose and insulin on body glycogen. Those alligators which received large amounts of

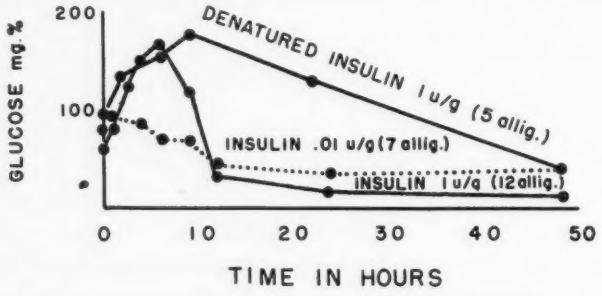

FIG. 3. Blood glucose content following the injection of commercial "insulin," and the injection of this "insulin" after incubation with 0.08 N KOH for 3 hours at 36°C.

glucose were injected repeatedly over a period of several days with glucose in amounts small enough to avoid exceeding the renal threshold. The blood glucose was maintained at a high concentration until the total amount had been administered. The procedure was the same for the groups which received insulin and glucose except for the injection of a single dose of insulin on the day before glucose administration. Glycogens were determined when the blood levels had returneed to the fasting value after the last injection of glucose. The results are presented in Table 1.

Although insulin reduces the blood glucose level effectively no dramatic effects of insulin on liver or body glycogen were noted in the few animals available for this study. Larger amounts of glucose seem to be necessary for the formation of extrahepatic glycogen than for the production of liver glycogen. It is to be hoped that in the future enough alligators of a size small enough to permit total glycogen studies will be available to enable us to continue a study of the mechanisms of gylcogen production.

The effect of adrenalin on blood glucose. Thirty alligators were injected with adrenalin to determine its effect on blood glucose. A typical experiment is shown in Figure 4. In contrast to the rapid hyperglycemic effect of the amorphous insulin preparation adrenalin has a delayed action. No rise in blood glucose occurs in the alligator for over two hours after intracardial injection of 2 cc. of 1/1000 adrenalin/kg. When the glucose rise does occur its height is greater than that produced by insulin or by the hyperglycemic factor uncontaminated within insulin. The delayed response to adrenalin is manifest in yet another way. About 2 hours after

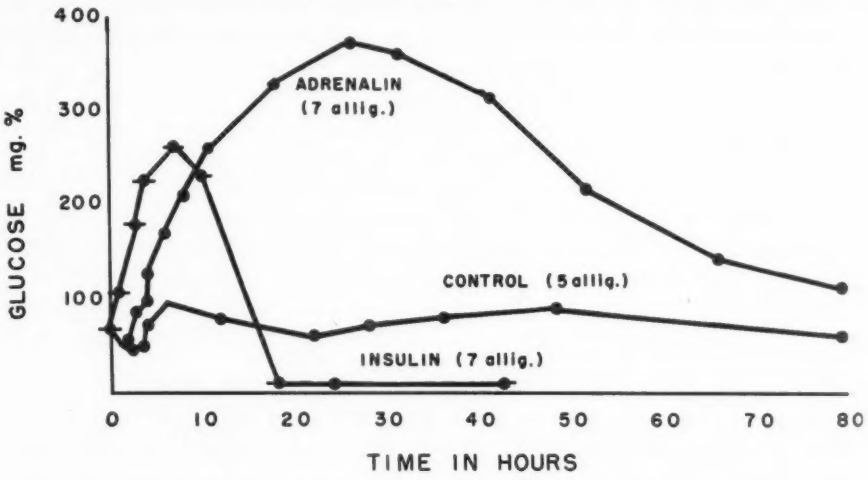

FIG. 4. Effect of adrenalin (2 cc. of 1/1000/kg.) and commercial "insulin" (1.0 U./gm.) on blood glucose.

injection the pupils of the eyes begin to constrict (a curious phenomenon), by three hours they are constricted to slits and for a period of several hours the animal is nearly blind. The time at which the pupils begin to constrict corresponds roughly to the time required for the blood glucose to begin to rise. The blood glucose produced by glycogenolysis through the action of either adrenalin or the hyperglycemic factor of amorphous insulin disappears at approximately the same slow rate as that observed following an equivalent dose of exogenous glucose. It would appear that once glucose appears in the blood the admittance of glucose into the cells in any form is attended with difficulty unless considerable amounts of insulin are administered.

If adrenalin is necessary to prepare the animal to meet emergencies it seems odd that the classic concept of an immediate pupillary reaction and an immediate rise in blood glucose does not hold in the alligator. If the con-

stricted pupils may be used as an index of the length of time that injected adrenalin remains active, this time seems to be of the order of 10 hours. We have not noted any consistent hyperglycemia which might be attributed to the production of adrenalin by the alligator's adrenals following handling the animals, injecting them with water, or withdrawing periodic samples of blood in scores of experiments. The controls in Figure 4 are representative.

The effect of adrenalin on body glycogen. An experiment was designed to determine the source of the blood glucose which increases after an intracardial injection of adrenalin. Five alligators which were given 2 cc. of 1/1000 adrenalin/kg. were killed when the glood glucose was judged to be

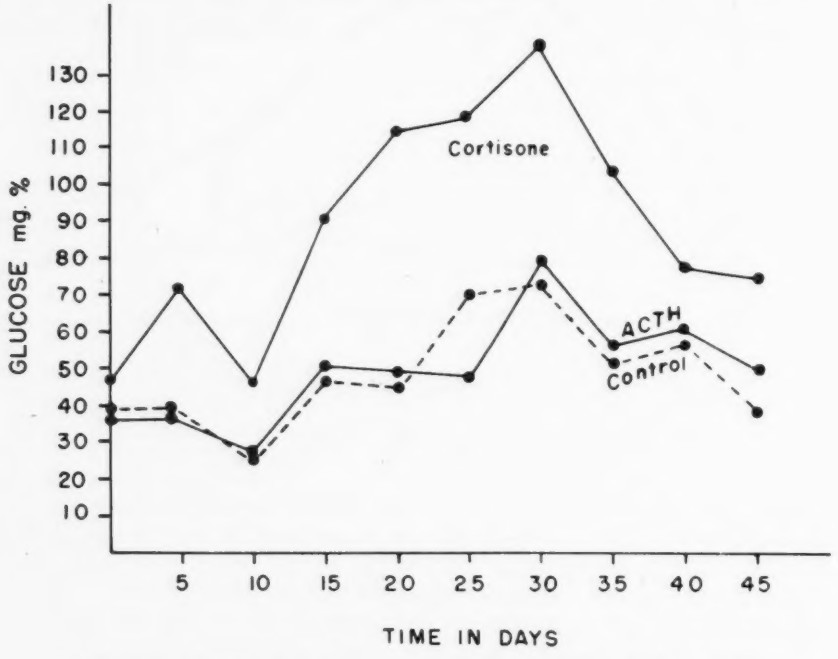

FIG. 5. Effect of daily injections of cortisone (10 mg./kg.) and ACTH (5 mg./kg.) on blood glucose. Five alligators were used in each group.

maximal at about 24 hours after injection and the liver and body were analyzed for glycogen. Over half the total body glycogen seems to have been lost from the stores by this procedure since the apparent glycogen depletion is approximately equal to that which would be needed to furnish the greatly increased blood glucose. Although the number of alligators available for this experiment was small it would appear that adrenalin

caused the hydrolysis of a remarkable amount of glycogen in the 24 hour period. In contrast to the mammals the alligator allows one a full day to observe the effect of either adrenalin or insulin on body glucose and glycogen.

The effect of cortisone and ACTH on glood glucose. Five alligators received 450 mg./kg. of cortisone divided into 45 daily intramuscular injections, 5 received 225 mg./kg. of ACTH similarly injected and 5 served as controls. Blood glucose content was determined daily in the 3 groups. Since the daily individual variations were considerable each of the points in Figure 5 represents an average of 5 days for the alligators in each group. If it is possible to draw any conclusions from this many animals cortisone caused an elevation of blood glucose which must be considered moderate in the light of the customary exaggerated response of the alligator to hyperglycemic agents. ACTH was ineffective in the dose administered. At the termination of the experiment on the 45th day blood was removed from the heart of each of the alligators and pH, CO_2, Na, K and Ca were determined. No significant variations from the normal were noted in the blood electrolyte patterns of those animals receiving etiher ACTH or cortisone. Caution should be exercised in interpreting the results of this experiment since the effective dose in the alligator may be much greater than that administered.

DISCUSSION

The picture presented of glucose metabolism is qualitatively similar to that of the higher mammals but quantitatively very dissimilar. The fact that the rate of all metabolic reactions is slowed down is both an advantage and a disadvantage. Although the use of the alligator makes it possible to separate reactions which would appear to be simultaneous in a mammal it is also true that many simple experiments on metabolic functions are so time consuming that the investigator is forced to proceed at a painfully slow pace. Many reptilian experiments have been abandoned by investigators who were unaware of the need to increase the observation time for almost every metabolic experiment to 10 times that required in mammalian work. In the experiments on the effect of the injection of adrenalin intracardially, the absence of any response for over two hours could easily convince a tired investigator that adrenalin has no effect on an alligator. Both adrenalin and the hyperglycemic contaminant of insulin seem to produce glycogenolysis. The mechanism is believed to be very similar and yet crude insulin is active in a matter of minutes after an intramuscular injection whereas adrenalin shows little to no activity before 2 hours. It is hoped that experiments now in progress may help to clarify some of these problems.

SUMMARY

1. Injected glucose is removed from the blood stream of the alligator at a very slow rate. This rate is determined by the temperature of the animal and is a function of the metabolic rate.

2. Glucose space in the intact alligator is similar and may be identical in volume with thiocyanate space.

3. One unit of "insulin"/gm. body weight produces an immediate state of "shock" which lasts a few hours. This condition occurs during the period of hyperglycemia. A second state of "shock" which is due to hypoglycemia follows more than a day later.

4. Large doses of "insulin" produce prolonged hyperglycemia due to the contamination of the commercial product with the hyperglycemic factor.

5. Large amounts of glucose promote the formation of liver and body glycogen.

6. For 2 hours after the injection of adrenalin no effects on blood glucose or pupillary response were observed. After 2 hours the pupils constrict to slits and the blood glucose rises several hundred mg.%.

7. Adrenalin reduces the glycogen stores of the liver to about $\frac{1}{3}$ the normal value and the body glycogen to about $\frac{1}{2}$ the normal in 24 hours.

8. Prolonged daily injection of 10 mg. cortisone/kg. into 5 alligators caused a moderate hyperglycemia. Daily injections of five mg. ACTH/kg. had no effect.

9. Neither ACTH nor cortisone in the amounts listed above had any noticable effect on electrolytes.

Acknowledgements

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ESTRONASE ACTIVITY OF HUMAN AND RODENT TISSUES[1]

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THE conversion of estrone, by minced uterus and to a lesser degree by heart, lung, spleen and kidney, to a product possessing increased biologic activity was originally reported by Heller (1940). It was later observed in this laboratory (Bischoff, Katherman and Yee, 1951) that mammalian red cells also possessed the property of converting estrone to a more powerful estrogenic substance and the enzymatic nature of the reaction was established (Bischoff, Katherman, Favati and Gray, 1952). The work of Heller was performed with rat and rabbit tissues. The present study includes the determination of "estronase"[2] in human tissues, investigates the activity in rodent tissues not previously studied, and places the tissues already studied upon a quantitative rather than a qualitative basis.[3]

PLAN

The species studied were man, mouse, rat and rabbit. The estronase activity was measured by the procedure previously described for red cells (Bischoff *et al.*, 1952), which differs from Heller's in that 100 to 200 μg. estrone per 1 gm. tissue were used instead of 6 μ. The increase in substrate was accomplished by employing 6% bovine albumin, in which estrone is soluble (Bischoff and Katherman, 1952), as the dispersing agent

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[1] This investigation was supported (in part) by research grants (C 1176 C, C 1587) from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

[2] Since the enzyme system (or systems) which converts estrone to a more powerful estrogen (Bischoff *et al*, 1951, 1952) may be nonspecific, some objection has been raised to labeling the enzyme "estronase". Regarding the enzymatic nature of the system, there can be no doubt (Bischoff *et al*, 1952) and we, therefore, use the expression estronase activity to label the phenomenon, although the exact role that estrone plays (substrate, coenzyme or acceptor) is necessarily obscure.

[3] In the present study 8 pair of litter mates are the fewest number of rats upon which any value is reported, excepting human blood for which 6 or 7 pair were used. The standard deviations of the means indicate that a recovery value deviates not more than 15% 19 of 20 times.

instead of Ringer's solution. The albumin solution has the advantage of being bacteriostatic and the increase in substrate concentration imposes a more severe criterion[4] for enzyme activity. The bioassay was performed by the method previously described (Bischoff *et al.*, 1951; Bischoff and Pilhorn, 1947) in which the uterus of the immature rat serves as the objective measure. Heller used a similar procedure.

In order to make the data comparable, the results are reported as an estronase index, which is the percentage increase in biologic activity resulting when 1 gm. tissue acts during 2 hours upon 200 μg. estrone at 38° C.

RESULTS

A summary of the results is given in Table 1.

The lowest estronase activity is observed in the muscle, whether skeletal, cardiac, or uterine. Heller reported some activity in muscle, while our results are essentially negative. In interpreting this apparent discrepancy it should be remembered that Heller's substrate concentration was one-twentieth that used by us and blood, which at that time was not known to have estronase activity, may not have been completely removed.

In studying the uterus, the whole organ was tested in the case of the laboratory animals; in man the endometrium and myometrium were studied separately. For the mouse a pooled sample from 12 animals, 30 days postpartum, was taken, The value for the rat is calculated from Heller's data for the pregnant rat. He states the value for the nonpregnant rat is lower. The estronase levels of the rodent uteri studied are uniformly high, much higher than that of man in which the endometrium has the same order of activity as the whole uterus of the rodent. It should be noted that added glucose was not required for the uterine reaction and NaF had no inhibitory effect.

The estronase activity of the rodent lung was found to have the same order as that of the uterus. Added glucose was not required for the reaction, but in contrast to the uterus, NaF had a marked inhibitory effect (index of 41 ± 6 against 87 ± 10 for the control).

The estronase reaction had not been previously studied for the testes and the results offer a striking and rather perplexing figure. In the rodent the testes have the same (or higher) order of activity as the uterus. The rabbit testes show the highest level of any tissue studied. In man (age

[4] When the concentration is of the order below 10 gamma per cc., the possibility of a nonenzymatic stoichiometric reaction with the more powerful reducing and oxidizing agents, which are present in cellular constituents in this range, cannot readily be ruled out.

TABLE 1. ESTRONASE INDEX OF MAMMALIAN TISSUES

Tissue	Mouse	Rat	Rabbit	Man
Skeletal muscle	6 ± 4	0 ± 2[1]	2 ± 7	
Cardiac muscle			-1 ± 8[1]	
Myometrium				7 ± 3
Endometrium				24 ± 8[5] 93 ± 12[5]
Leiomyoma				14 ± 7 6 ± 3
Uterus	40 ± 9[2]	240[6]	153 ± 13 60 ± 15[1,2]	
Lung	87 ± 10[1,3]	59 ± 10	95 ± 11	
Testes	209 ± 14[3]	134 ± 6	859 ± 79[4] 544 ± 42[1]	9 ± 9[7] 102 ± 10[1,3,8]
Gastric mucosa				33 ± 4
Thyroid				10 ± 4
Prostate hypertrophied				3 ± 3
Red blood cells			39 low 64 mean[10] 98 high	15 low 21 mean[9] 45 high

[1] Added glucose to tissue previously washed with saline, was without effect.
[2] No effect of NaF in a concentration of 5 mg. per cc.
[3] Marked inhibitory effect of NaF in a concentration of 5 mg. per cc.
[4] Marked inhibitory effect of methylene blue in a concentration of 0.5 mg. per cc.
[5] Secretory phase.
[6] Calculated from Heller's data.
[7] Testes of man aged 73, showing marked degenerative changes grossly.
[8] Testes of man aged 47.
[9] Nine determinations upon blood samples of 9 females (man).
[10] Eight determinations upon blood samples of 8 rabbits.

47) the testicular activity is of the same order as that of the human endometrium. The results for the degenerative testes of a man aged 73 were negative. Like the uterus the testes required no added glucose for the enzymatic reaction, but in contrast NaF had a marked inhibitory effect (42 ± 8 versus 86 ± 10, 65 ± 9 versus 209 ± 14). Methylene blue had an inhibitory effect upon the testes reaction as it does for red cells (316 ± 25 versus 859 ± 79, 0.5 mg. methylene blue per cc.).

In man the thyroid and prostate showed a low order of activity, the gastric mucosa an intermediate range.

The values for human red cells are taken from 9 female human bloods, age 20 to 82, to establish the range. Human red cells have about one-third the estronase activity of rabbit red cells.

DISCUSSION

The estronase reaction in blood requires glucose or some related compound (Bischoff *et al.*, 1952). That added glucose is not required to reestablish the enzyme reaction in testes, lung, or uterus, washed free of endogenous glucose, does not necessarily indicate a different enzyme system from that in blood, since the necessary compound may be liberated on incubation. It is well established that the testes and uterus carry glycogen and this may serve as the carbohydrate reservoir.

The difference in the behavior of specific tissues to fluoride is not readily explained, but is nevertheless characteristic for each tissue.

The high estronase activity of the lung and testes and the moderately high activity of the gastric mucosa diminish the significance of the implications in regard to the high level originally reported for the uterus. The significance of the high level for this group of tissues remains at present obscure. It is interesting that both the endometrium and testes activate estrone, but the end result of exogenous estrogen is quite the opposite, producing hypertrophy in one, atrophy in the other.

The distribution of estronase activity over so many tissues, including the red cells, would indicate that estrone *per se* would have little chance of transportation in the body. We found originally (Bischoff *et al.*, 1952) that estrone sulfate does not suffer enzymatic activation by the red cells and this is probably the form in which estrone is transported.

It is not improbable that estrone *per se* is devoid of biologic activity and that it is the reaction product which is estrogenic.

SUMMARY

The estronase activity of muscle, red cells, uterus, lung, and testes follows a similar pattern in man and the rodents.

The very high activity of the testes, not hitherto reported, is found in all species studied.

The range of estronase activity for the red cells of 9 female human bloods was ascertained.

NaF has a marked inhibitory effect upon the estronase reaction in lung and testes but not in the uterus.

ADDENDUM

Ryan and Engel (Endocrinology 52: 289, 1953) have recently demonstrated the conversion of estrone to estradiol by the human testes. They recovered 47% of the estrone added. Seven per cent estradiol was identified. Goldzieher and Roberts had previously (Clinical Endocrinology and Metabolism 12: 143, 1952) identified endogenous estradiol in human testes. In our bioassay procedure estradiol has twenty times the activity

of estrone; the conditions of the Ryan and Engel experiment, assuming the unidentified fraction of 46% is nonestrogenic, would have given a recovery of 187% as estrone equivalents. In the experiment for human testes recorded in this paper the recovery was 355% indicating either that more estradiol was produced (15%) or that the unidentified fraction is possessed of biologic activity. For rabbit testes we recovered 959% as estrone equivalents, which would indicate a conversion of 45% to estradiol, if estradiol is assumed to be the active reaction product.

Acknowledgment

The authors are indebted to Dr. Vittorio Favati for assistance in the bioassay, to Drs. E. L. Benjamin, John P. Blanchard, D. R. Dickson, W. R. Flood, and Kathryn L. Stephenson for specimens, and to Parke, Davis and Company for the estrone.

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THE ACCELERATING EFFECT OF ADRENALECTOMY ON REGROWTH OF HAIR IN THE RAT: REGROWTH OF HAIR IN PARABIOTIC RATS, ONE PARTNER BEING ADRENALECTOMIZED

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IN a previous paper (Zeckwer, 1953), it was shown that the accelerating effect of adrenalectomy on regrowth of hair in the rat does not depend on the presence in the anterior pituitary of granulated acidophils. Thus in thyroidectomized rats with pituitaries devoid of acidophil granules there was accelerated regrowth of hair following adrenalectomy, although such regrowth had a longer lag period than in adrenalectomized rats with thyroids intact. Evidence was presented that the regrowth of hair after adrenalectomy might be due to an increased secretion by the pituitary of somatotrophic hormone (STH). Whatever the growth promoting substance may be, and, in particular, whether or not it is STH, remains for future work to reveal. In the meantime it is of interest to find out whether the substance promoting hair growth is sufficiently increased in amount after adrenalectomy to pass across to a parabiotic partner.

To answer this question rats were united in parabiosis, one partner being adrenalectomized, the other partner having adrenals intact. The parabiotic technique is useful in demonstrating excess production of an endogenous secretion in one partner which in the case of many secretions (follicle stimulating hormone, adrenocorticotrophic hormone, etc.) passes over to the other partner. This subject has been recently reviewed (Finerty, 1952).

METHODS

Wistar albino rats of a closely inbred strain, of the same sex and of the same litter were united in parabiosis, and sufficient time allowed after operation to insure that no incompatibilities existed between the partners (Zeckwer, 1952). The parabiotic operation consisted of suturing together the muscles of lateral abdominal walls and peritoneum, without open communication between peritoneal cavities. Following this, long lateral skin incisions were sutured together. One partner was adrenalectomized in some experiments before, usually after parabiosis. Sodium chloride drinking fluid (1%) was given for several weeks while records of hair growth were made. The rats were fed Purina dog chow.

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Ten pairs were studied, 10 to 89 days after adrenalectomy. Controls consisted of parabiotic rats with adrenals intact, single adrenalectomized rats on NaCl drinking fluid, and single normal rats, in some of which sham operations had been performed.

Hair was shaved by electric clippers from the back and sides of all rats of an experimental group; regrowth of hair was recorded by drawings and photographs. After data were obtained, the rats were reshaved and regrowth was again studied.

Since it is known that the cycles of hair growth are slower in older rats, fairly old rats were used in these experiments. They varied from 115 to 277 days of age at time of adrenalectomy.

RESULTS

No changes in hair growth occurred during the first 2 weeks after adrenalectomy. After this initial lag, the adrenalectomized rats, whether single or in parabiosis, showed rapid diffuse growth of hair, while the non-adrenalectomized rats, single or in parabiosis, showed the slow regular patterns of regrowth, consisting of patches of long hair alternating with completely bald areas, the distribution depending upon the time in the cycle at which the regrowth was observed. Thus in a parabiotic pair, the adrenalectomized partner, with rapid diffuse regrowth of hair, did not cause enhanced growth of hair in the intact partner. It was concluded that no growth accelerating factor passed across the parabiotic union.

The parabiotic operation caused a slight delay in the appearance of the diffuse hair growth in the adrenalectomized partner in comparison to the growth in the single adrenalectomized animals.

After results were recorded, salt was withdrawn from the drinking fluid in 9 pairs. The single adrenalectomized rats soon developed adrenal insufficiency when no saline was given, but 7 of the adrenalectomized rats united to intact rats survived, the intact rat maintaining the adrenalectomized partner. This is consistent with the findings of Jacobsohn (1949), Copp and Finerty (1950), and Westman and Jacobsohn (1944). The adrenals of rats united with adrenalectomized partners show increased growth, according to Li and P'an (1940) and Houssay and Pinto (1944). One explanation for this is that ACTH from the adrenalectomized parabiont passes over to the normal partner. That the adrenalectomized parabiont is maintained by the salt content of the blood from the normal partner is an explanation suggested by Jacobsohn (1949).

In some instances the adrenalectomized partner became the larger rat. This may be because nutrients received from the normal partner compensated for the decreased food intake known to occur in the adrenalectomized animal. This supports the evidence discussed in the previous paper (Zeckwer 1953) that adrenalectomy leads to increased rate of secretion of growth hormone STH, an effect which is usually masked by decreased food intake. On the other hand, the discrepancy in somatic growth between the partners may be due to reduced growth of the intact rat with stimu-

lated adrenals, since it is known that adrenal cortical hormone suppresses somatic growth.

At late intervals, following the withdrawal of salt, some of the intact partners showed retarded hair growth, as though their adrenals were increased in activity (Fig. 1). It is known that an excess of adrenal cortical hormone suppresses hair growth. At the same time, three of the adrenalectomized partners ceased to show the accelerated diffuse growth of hair, but resumed the normal pattern of hair growth in patches alternating with bare areas, as though the adrenalectomized parabionts were receiving adrenal cortical hormone from their partners.

In two pairs that survived withdrawal of salt, the partner with adrenals intact was ovariectomized. It was found that follicle stimulating hormone passed from the ovariectomized rat to the adrenalectomized partner, thus proving the adequacy of the circulation in the parabiotic union.

The union between 5 pairs of parabionts was severed surgically and the rats allowed to live as separate individuals. When no salt solution was given, the adrenalectomized rats developed adrenal insufficiency.

DISCUSSION

If the adrenalectomized partner was producing a considerable excess of some substance which stimulated hair growth, one might anticipate that this growth factor would pass to the partner, unless the substance had a high clearance rate and was utilized very quickly in the animal in which it is produced (Huff *et al.*, 1950; Lucké *et al.*, 1952).

Van Dyke *et al.* (1950) have shown that injected growth hormone of the pituitary can pass the parabiotic union. It has been shown by Bucher, Scott, and Aub (1951) and by Wenneker and Sussman (1951) that when a partially hepatectomized rat is united in parabiosis with a normal rat, a growth factor passes over from the hepatectomized rat and causes in the intact partner an increase in growth of hepatic cells. In the present experiments the absence of any demonstrable increase in hair growth of the normal rat united with the adrenalectomized rat suggests several possibilities:

Explanation of Figure

Fig. 1. Litter-mate male rats 18 days after shaving. (a) Normal intact rat showing symmetrical patches of hair growth. (b) Rats in parabiosis 39 days. The lower (left) partner, adrenalectomized 32 days previously, shows diffuse growth of thick hair. The upper (right) partner has intact adrenals. It shows no acceleration of hair growth, but on the contrary shows diminished regrowth of hair, attributed to probably increased adrenal cortical activity. This pair survived subsequent withdrawal of salt. After surgical separation, the adrenalectomized rat died. (c) Rat adrenalectomized 32 days previously shows diffuse thick hair similar to lower partner of (b). This rat did not survive subsequent withdrawal of salt.

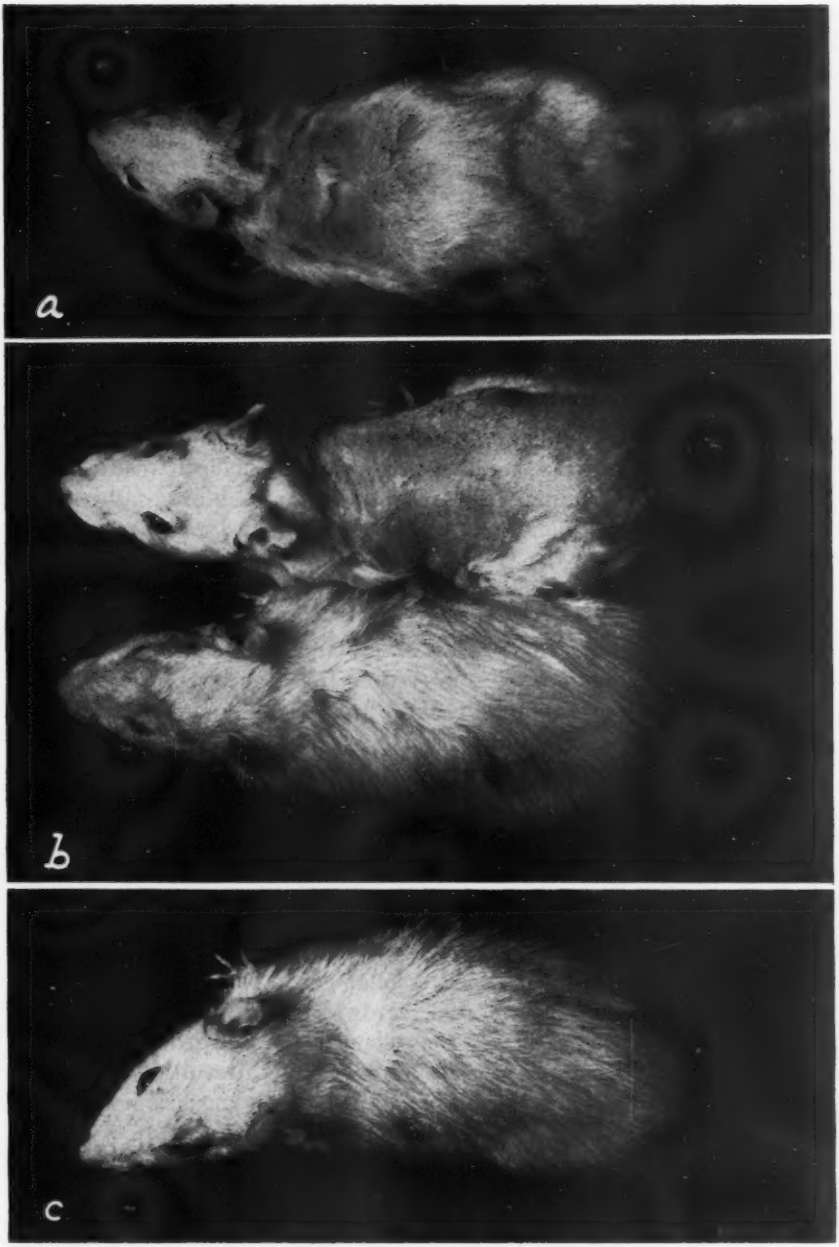

FIG. 1.

(1) the adrenalectomized rat is not producing growth factor in sufficiently increased amounts to pass the parabiotic union; (2) the growth factor is a substance that can not pass the union; (3) the growth factor is opposed by the depressing effect of increased adrenal cortical activity in the intact partner; (4) the growth factor is immediately utilized by the adrenalectomized rat, i.e., has a high clearance rate, so that no excess remains to pass to the other rat. It is known that certain hormones, like estrogen, do not pass across the parabiotic union unless in very large amounts. This is probably because estrogen is immediately utilized by the tissues, just as thyroid cells immediately fix thyroid stimulating hormone (TSH) so that TSH disappears rapidly from the blood. It is only when there is no target organ, that is thyroid gland, that TSH can be demonstrated in excess.

It therefore seems that the fourth hypothesis is reasonable. In contrast to the present experiment is the situation in the case of an ovariectomized rat united with a normal. Here follicle stimulating hormone (FSH) of the ovariectomized rat has no target organs to utilize it and it readily passes across the parabiotic union. In the case of adrenalectomy, if there is an increase in growth hormone, many cells of the body probably are target organs to fix or utilize the trophic substance, and there would be little likelihood of endogenous growth hormone being present in the blood in excess of its utilization.

In the experiments of Van Dyke *et al.* (1950) the amount of growth hormone injected may have been greater than the capacity of the tissues to utilize it, and therefore represents more than the amount of endogenous growth hormone that the pituitary is likely to secrete after adrenalectomy.

SUMMARY

Ten pairs of litter-mate rats of the same sex were united in parabiosis. One partner was adrenalectomized at varying periods before or after parabiosis. Controls consisted of single intact, single adrenalectomized, and intact rats in parabiosis with intact rats. Hair was shaved from back and sides of the rats and regrowth recorded.

The adrenalectomized rats, whether single, or in parabiosis with normal partners, showed diffuse accelerated regrowth of hair. The intact partner of the adrenalectomized parabiont showed no accelerated growth. This gives evidence that the substance promoting hair growth resulting from adrenalectomy is either not in sufficient quantities to pass the parabiotic union, or is a substance that can not pass the parabiotic union, or its effect is opposed by increased adrenal cortical activity in the intact partner, or is immediately utilized by the tissues so that no excess remains in the circulation. The latter hypothesis is suggested as a reasonable explanation.

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THE EFFECT OF PROPYLTHIOURACIL ON RADIOIODIDE CONCENTRATING BY THE THYROID GLAND IN NORMAL AND HYPOPHYSECTOMIZED MICE

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THE ratio of the iodide concentration in the thyroid gland to that in serum (T/S ratio) has been measured in the rat (VanderLaan and VanderLaan, 1947; Taurog, Chaikoff and Feller, 1947; VanderLaan and Greer, 1950), mouse (Wollman and Scow, 1953), man (Stanley and Astwood, 1949) and chick embryo (Wollman and Zwilling, 1953). The three different methods of measuring this ratio which were used in the above studies can be described as follows:

1. The concentration of total stable iodide in the thyroid gland is compared with an estimate of the iodide concentration in the serum (Taurog, Tong and Chaikoff, 1951).

2. Using radioiodide, the concentration of thyroid iodide that is freely exchangeable with serum iodide is compared with the concentration of serum iodide under two sets of conditions:

a. When organic binding of thyroid iodide is permitted (Wollman and Scow, 1953).

b. When organic binding of thyroid iodide is blocked by administration of a suitable goitrogen (VanderLaan and VanderLaan, 1947; Taurog, Chaikoff and Feller, 1947; Wollman and Scow, 1953). Values for T/S ratios obtained with the various methods differ. Since there are no reports in which at least two of the methods were used in duplicate groups of animals, it is difficult to explain the discrepancies. It is possible that the discrepancies in results were due to differences in endogenous production of thyroid stimulating hormone in the groups of animals being compared, or incomplete equilibration of radioiodide between thyroid gland and blood in situations requiring equilibration (Gross and Pitt-Rivers, 1952).

This paper reports measurements of the ratio of the concentrations of thyroid radioiodide (which is freely exchangeable with serum iodide) and serum radioiodide for cases where organic binding of iodide was blocked and where binding was permitted in duplicate groups of animals. This has been done for both normal and hypophysectomized mice.

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It was found that in normal mice the T/S ratio was approximately 250 when organic binding was blocked. For its maintenance, this large concentration differential of radioiodide between thyroid gland and blood appeared to require the continued circulation of blood through the thyroid gland. In hypophysectomized mice the T/S ratio was larger when organic binding of iodide by the thyroid gland was blocked than when binding was permitted. Although this effect of organic binding on the T/S ratio was not demonstrated in normal mice it cannot yet be excluded.

METHODS AND MATERIALS

Measurements were made on male C3H mice, three to four months of age. They had been fed a pelleted stock diet of moderately low iodine content (Wollman, Scow, Wagner, and Morris, 1953) starting from the age of seven weeks. Mice were at least 12 weeks of age when they were hypophysectomized through the parapharyngeal approach. Completeness of the operation was ascertained by visual inspection of the region of the sella turcica for pituitary fragments using a binocular microscope at 9× magnification.

The T/S ratio was determined by injecting radioiodide, allowing time for equilibration of the radioiodide and then measuring the radioiodide concentration in the thyroid gland and in the serum.

Blocking of organic binding: Organic binding of radioiodide was prevented by a subcutaneous injection of 2.5 mg. of propylthiouracil dissolved in 0.5 ml of a 0.9% NaCl solution which contained NaOH at a concentration of 0.04N. This was administered at least one-half hour before injection of I^{131}. The test to demonstrate that blocking was complete was made by homogenizing thyroid glands of experimental mice and then measuring the fraction of the total I^{131} in the gland which remained in the supernatant after deproteinization (non-precipitable iodine).

I^{131} injections: In general, 10 to 20 μc of I^{131} [2] was given in the form of carrier-free NaI. An aliquot of stock radioiodide solution was diluted with distilled H_2O and usually 0.5 to 1.0 ml of solution was injected intraperitoneally into each mouse.

Procedure at autopsy: At autopsy, each mouse was anaesthetized with ether, the thoracic cavity opened, and blood taken from the heart. The thyroid gland was then removed. In the mice not treated with propylthiouracil, immediately after removal the thyroid was chilled or frozen by placing it in contact with a clean surface of dry ice (solid CO_2), and then weighed. In one experiment, however, the thyroid gland was removed immediately after anaesthetization and before the bleeding. In mice treated with propylthiouracil, the thyroid gland was removed immediately after exsanguination, weighed, and then placed into a sample vial or into a homogenizer. Samples from each mouse were analysed separately.

Deproteinization: After cooling and weighing, the thyroid glands were put into individual glass homogenizers containing 6 ml of ice cold distilled water and one mg of NaI as carrier, and were homogenized immediately. The homogenate was deproteinized by adding to it 0.10 ml each of the $Ba(OH)_2$ and $ZnSO_4$ solutions of Somogyi (1945), followed by centrifuging and removing the supernate with a pipette. In a few experiments trichloracetic acid (TCA) was used as the deproteinizing reagent, but only to compare completeness of deproteinization with that using Somogyi's precipitant.

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[2] The I^{131} was supplied by the Oak Ridge National Laboratory, Oak Ridge, Tennessee.

Serum was deproteinized by diluting a 0.05 ml sample to 0.80 ml with water containing 0.1% NaI and adding to it 0.10 ml each of the $Ba(OH)_2$ and $ZnSO_4$ solutions, as above.

I^{131} determinations: Precipitable and non-precipitable I^{131} as well as total I^{131} in thyroid glands were measured by gamma counting.

Serum I^{131} content was determined by measuring, with an end-window Geiger tube, the I^{131} in an aliquot of diluted serum (0.05 ml diluted to 1.00 ml with H_2O containing 0.1% NaI) which was dried on a copper planchet. A similar technique was used on samples from deproteinized serum. Self-absorption corrections were found to be negligible under the conditions of measurement. Statistical error of counting was less than 3%.

RESULTS

1. *The T/S ratio when organic binding of iodide was blocked*

Proper determination of the T/S ratio when binding is blocked requires that several important factors be considered: 1) The dose of propylthiouracil used (2.5 mg.) must prevent the binding of administered radioiodide by the thyroid gland; 2) radioiodide should not be lost from the thyroid gland in the time between taking the blood sample and excising the gland; 3) equilibration of radioiodide between thyroid and serum should be attained.

Effectiveness of propylthiouracil in blocking binding: Radioiodide was injected into four mice one hour after administration of 2.5 mg. of propylthiouracil. Four hours after the radioiodide injection, an average of 98 percent of the thyroid I^{131} remained non-precipitable when the homogenate of the thyroid gland was deproteinized.

Loss of thyroid I^{131} after death: Since the I^{131} is not protein bound in the propylthiouracil-treated animals an investigation was made to see if there was a loss of I^{131} from the thyroid gland following death. Beginning at 90 minutes after the radioiodide injection, normal mice were killed by exsanguination. At various time intervals, up to 80 minutes after exsanguination, thyroid glands were excised and their I^{131} content was determined. Five mice were used for each time. It was found that after death the ratio of the concentration of thyroid radioiodide to serum radioiodide concentration decreased progressively with increasing time intervals after death (Fig. 1). When the thyroid was removed 80 minutes after exsanguination, the T/S ratio was only one-third of that obtained when the thyroid gland was excised immediately after exsanguination. The shape of the curve indicates that non-precipitable I^{131} starts leaving the thyroid gland immediately after death. However, the initial rate of loss was slow enough so that little is lost if the thyroid is removed immediately after exsanguination.

Equilibration: The T/S ratio was measured at various time intervals after I^{131} injection in both normal and hypophysectomized mice. In a

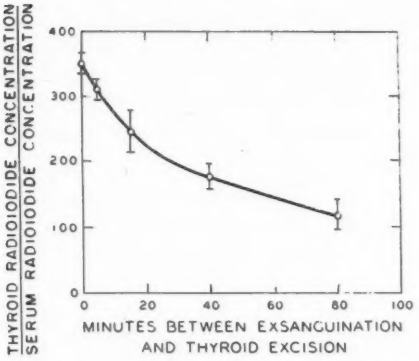

FIG. 1. Loss of radioiodide from the thyroid gland after death in normal mice treated with a single dose of propylthiouracil to prevent organic binding of radioiodide. The vertical line through each point represents the standard error.

preliminary experiment, it was found that in normal mice the ratio of the I^{131} concentration in the thyroid to that in blood at ten minutes after I^{131} injection was only 25% of that at 20 minutes, while the ratio at 20 minutes was only 65% that at 80 minutes.[3] In the final experiment, using six mice per point, equilibration in normal mice seemed to be reached by $\frac{1}{2}$ hour (Fig. 2). In contrast, in hypophysectomized mice 10 days after operation

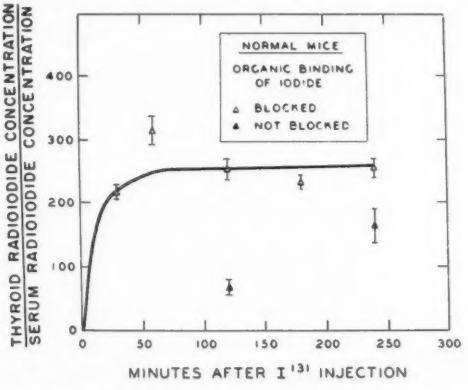

FIG. 2. Equilibration of radioiodide between thyroid gland and serum in normal mice. The vertical line through each point represents the standard error.

[3] The increase in the T/S ratio during the first 10 minutes after intraperitoneal injection was much less than the increase during the second 10 minute interval. This may be due to the fact that it takes approximately 10 minutes for the radioiodide concentration in the blood to reach its maximum following an intraperitoneal injection of radioiodide.

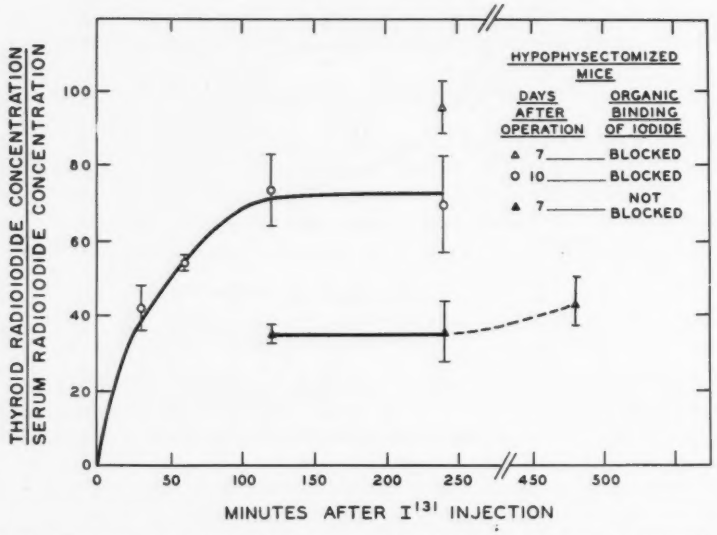

FIG. 3. Equilibration of radioiodide between thyroid gland and serum in hypophysectomized mice. The vertical line through each point represents the standard error.

(five or six mice per point) equilibration was not reached at one hour but was reached by two hours after I^{131} injection (Fig. 3). The T/S ratio in normal mice averaged 250 while the ratio in hypophysectomized mice averaged 70.

The radioiodide concentration in the blood at 240 minutes after I^{131} injection was more than half that at 30 minutes for both normal and hypophysectomized mice.

2. *The T/S ratio when organic binding of iodide was permitted*

Proper determination of the T/S ratio when binding is permitted requires that several important factors be considered: 1) Thyroid *radioiodide* must be measured, i.e., there must be good deproteinization; 2) organic binding of radioiodide should not continue after exsanguination; 3) serum radioiodide should not be contaminated by protein-bound I^{131}; 4) equilibration of radioiodide between thyroid and serum must be attained.

Deproteinization: A comparison was made between Somogyi's solutions and 8% trichloracetic acid (TCA) as protein precipitants. Duplicate aliquots of individual homogenates of thyroid glands of seven mice which had been injected with radioiodide were deproteinized with the two precipitants. Approximately twice as much of the thyroid I^{131} was found to be non-precipitable with TCA (4%) as with Somogyi's precipitant (2%).

There appear to be two possible explanations for this observation: a) TCA does not precipitate protein-bound I^{131} as completely as Somogyi's precipitant, or b) the precipitate formed by Somogyi's precipitant adsorbs ordinarily soluble radioiodide. It appears that the first alternative is the correct one. Supernatants (the fraction containing the non-precipitable I^{131}) from homogenates in TCA were slightly turbid after centrifugation, whereas those from homogenates in distilled water followed by deproteinization with Somogyi's precipitant and centrifugation were clear. In addition, the precipitate with Somogyi's precipitant did not adsorb non-precipitable I^{131}. In order to demonstrate the latter, thyroid glands from four mice not treated with I^{131} were added to individual solutions of radioiodide and carrier. After homogenization and deproteinization, 97 percent of the I^{131} was found to be non-precipitable.

Organic binding of iodide after thyroid excision: It seems improbable that appreciable organic binding of radioiodide continues after exsanguination or after excising the thyroid gland. In order to determine if any organic binding of I^{131} occurred in the thyroid gland during the time interval between exsanguination and thyroid excision, the following experiment was conducted. The T/S ratio was determined in two groups of six mice, one bled before and the other bled after thyroid excision, at one and one-half hours after radioiodide injection. It was found that the T/S ratio was 50 ± 6 in those bled before and 35 ± 2 in those bled after thyroid excision. This would indicate that the radioiodide concentration in the thyroid increased rather than decreased during the time interval between exsanguination and thyroid excision. Slightly less than half of the above difference in the T/S ratios appeared to be accounted for by the consistently larger thyroid weights observed (2.5 mg. vs. 2.2 mg.) when the thyroids were excised before exsanguination. The increased weight may be due to excess blood in and on the thyroid tissue. The remainder of the difference in the T/S ratios may be due to the liberation of radioiodide from organic bound radioiodine in the time interval between exsanguination and thyroid excision (Roche, Michel, Michel and Lissitzky, 1952).

The fact that 97% of the radioiodide added to I^{131} free thyroid glands just before homogenization was found in the supernate after deproteinization, as described above, suggests that there is little binding of iodide after excision of the thyroid gland.

In the following experiments, exsanguination was performed first.

I^{131} in serum: Two hours after radioiodide injection into normal mice, virtually all serum I^{131} was non-precipitable, but at 8 hours after radioiodide injection 39% of the serum I^{131} was precipitable. Since the precipitable I^{131} in serum probably was less at 4 than at 8 hours and the total I^{131} in blood at 4 hours was over 7 times the radioiodide content at 8 hours (Table 1), the use of total serum I^{131} as an estimate of serum radioiodide

TABLE 1. SERUM I^{131} AND PRECIPITABLE I^{131} IN THYROID GLAND

Group	Hours after I^{131} injection	Serum I^{131} (per cent of dose per gram)	Thyroid precipitable I^{131} (per cent of thyroid I^{131}
Normal	2	3.61 ±0.08[1](5)[2]	97.0 ±0.6(5)
	4	1.20 ±0.28 (5)	98.4 ±0.2 (5)
	8	0.161[3] ±0.007 (4)	98.6 ±0.2 (5)
Hypophysectomized[4]	2	6.95 ±0.45 (4)	44 ± 2 (4)
	4	5.98 ±0.40 (6)	71 ± 8 (6)
	8	2.37 ±0.32 (5)	89 ± 2 (5)

[1] Standard error of the mean.
[2] Number of determinations.
[3] Non-precipitable serum I^{131}.
[4] Seven days after hypophysectomy.

cannot be in error by more than 10%. In addition, incorporation of radioiodide into the thyroxine fraction of the thyroid gland has been reported to be rapidly and extensively decreased in mice after hypophysectomy (Wollman and Scow, 1953). For these reasons total serum I^{131} concentration was used in place of serum radioiodide concentration in both normal and hypophysectomized mice, except in normals at 8 hours after I^{131} injection.

Equilibration of radioiodide between thyroid gland and serum: The T/S ratio was determined at various time intervals after radioiodide injection in normal mice and in mice 7 days after hypophysectomy, using 5 or 6 mice per point (Figs. 2, 3). The T/S ratio in hypophysectomized mice was a constant independent of the time after injection, but in normal mice it increased between 2 and 4 hours after injection and reached a value of approximately 1000 at 8 hours after injection. Additional data on serum I^{131} and on the ratio of precipitable I^{131} to total I^{131} in the thyroid gland were obtained during the above determinations (Table 1). Since these data have bearing on the evaluation of the above results they will be discussed below.

DISCUSSION

When organic binding of iodide by the thyroid gland was blocked in normal mice the ratio of the radioiodide concentrations in the thyroid gland and serum (T/S ratio) was approximately 250. The maintenance of the major part of this large concentration differential appeared to be dependent on the continued flow of blood through the thyroid gland since the T/S ratio promptly began to decrease after exsanguination (Fig. 1). Several reviewers have noted (Means, 1951; Albert, 1952) that the maintenance of the concentration differential may involve either complexing of iodide with a substance present in the thyroid tissue, or a coupling with an energy yielding process in the thyroid. If iodide concentrating were due to an ion exchange type adsorption on a stable substance,

one would expect the thyroid radioiodide concentration to remain constant after death. The present observation might suggest that at least part of the iodide concentrating phenomenon is not due to an adsorption mechanism of this type. However, due to the complex changes that may take place after cessation of blood flow, *e.g.*, pH changes, it is not possible to conclude that the present experimental finding excludes or supports any of the suggested mechanisms.

A steady state in which the ratio of the radioiodide concentrations in thyroid gland and in serum was independent of the time after radioiodide injection was attained in hypophysectomized mice both when binding was blocked or permitted and in normal mice only when binding was blocked. On the other hand, when binding was permitted in normals the T/S ratio increased progressively with time. It is not possible to conclude however, that a steady state was not attained in this case since the increase in the T/S ratio may have been due to an error in the estimate of the thyroid radioiodide concentration. For example at 4 hours, when precipitable I^{131} made up almost 99% of the total thyroid radioiodine (Table 1), a small error of 1% in the completeness of precipitation of this I^{131} would render the non-precipitable I^{131} too high by 100%. As indicated by the data in Table 1, deproteinization cannot be less than 98.6% complete. The T/S ratios for the hypophysectomized mice are probably more reliable since less than 90% of the thyroid I^{131} was precipitable (Table 1). A deproteinization which was only 99% complete in this case would make less than a 10% error in the determination of the T/S ratio.

When organic binding was permitted in normal mice it was observed that at 2 hours after injection the non-precipitable thyroid I^{131} was only three percent of the total thyroid I^{131} (Table 1). This implies that the freely exchangeable iodide in the thyroid gland has been bound and replaced repeatedly by serum iodide. The decrease in blood radioiodide concentration during the first 2 hours after injection would be expected to cause a decrease in the thyroid radioiodide concentration and hence increase the ratio of precipitable to non-precipitable thyroid I^{131}. When corrections are made for this, the ratio of precipitable to non-precipitable thyroid I^{131} is large and indicates that there has been approximately 12 replacements of freely exchangeable thyroid iodide by serum iodide. If it is assumed that all freely exchangeable thyroid iodide is equivalent, the large number of replacements of thyroid iodide by serum iodide would suggest that a steady state had been attained, and that the observed increase in the T/S ratio was due to analytical difficulties as discussed in the preceding paragraph. Furthermore, it would suggest that when organic binding was permitted the T/S ratio was not over 60 (the value at 2 hours) and that it was significantly less than when binding was blocked. In view

of the 12 replacements if a steady state had not been attained, then it must be concluded that thyroid iodide exists in at least two compartments which exchange with blood iodide at markedly different rates. No evidence for two compartments has been observed when the T/S ratio was measured when binding was blocked.

The T/S ratio in hypophysectomized mice seven days after operation (Fig. 3) was significantly larger when organic binding of radioiodide was blocked than when permitted (96 as compared with 38). This effect is interpreted as follows: The thyroid radioiodide concentration is less when organic binding of iodide is allowed because thyroid iodide is removed by the organic binding process. As the time interval required for organic binding of iodide present in the thyroid tissue decreases relative to the time interval required for equilibration of thyroid iodide with serum iodide, the thyroid iodide concentration, and hence the T/S ratio, might be expected to decrease progressively (Wollman, 1953). This interpretation must be considered tentative because it assumes the sole action of the propylthiouracil is to prevent organic binding of iodide. It may be, of course, that propylthiouracil increases the iodide concentrating ability of the thyroid gland directly, and that measurements of the T/S ratio using it, although a measure of a characteristic property of thyroid tissue, have no simple relationship to the iodide concentrating ability of the gland in the untreated mouse.

The value of the ratio of the radioiodide concentrations in the thyroid gland and serum after a steady state is attained may not be the same as the corresponding ratio for total stable thyroid iodide, or even for freely exchangeable stable thyroid iodide. As shown elsewhere (Wollman, 1953), a simple theoretical calculation indicates that the T/S ratio for radioiodide administered by a single injection will be larger than the ratio for freely exchangeable stable thyroid iodide. In order that the difference be negligible it is necessary that the time interval required for the blood radioiodide concentration to be reduced by 63% (mean life) be long compared with the time interval required for the T/S ratio to reach approximately 63% of its steady state value. This condition appears to be reasonably well satisfied in those cases in which a steady state was reached. The time interval required for a T/S ratio to equal 63% of its steady state value was approximately 14 minutes in propylthiouracil-treated normal mice and approximately 50 minutes in hypophysectomized mice while the mean life for radioiodide in the blood in both cases was over 240 minutes. It therefore appears reasonable to expect the T/S ratio in these mice to be not appreciably larger than the corresponding ratio for freely exchangeable stable thyroid iodide.

SUMMARY

When organic binding of thyroid iodide was blocked by a single dose of propylthiouracil the ratio of radioiodide concentration in the thyroid gland to that in the serum was approximately 250 in normal mice. Maintenance of this high concentration differential required the continued flow of blood through the thyroid gland. Ten days after hypophysectomy the T/S ratio was reduced to approximately 70. When organic binding was blocked, equilibration of radioiodide between thyroid gland and blood was found to occur within approximately one-half hour in normal mice but required one to two hours in hypophysectomized mice 10 days after operation. The ratio in hypophysectomized mice appeared to be significantly less when organic binding was permitted than when blocked.

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NOTES AND COMMENTS

REPRODUCTIVE PERFORMANCE OF FEMALE MICE TREATED PREPUBERALLY WITH A SINGLE INJECTION OF ESTRADIOL DIPROPIONATE[1]

Female rats treated prepuberally with various estrogens have been reported to be deficient in reproductive ability at maturity (Greene and Burrill, 1941; Turner, 1941; Hale, 1944; Hale and Weichert, 1944). Wilson (1943) found that injections of estradiol dipropionate (12 injections totaling 1.2 mg. in 28 days), when begun at one, five or ten days of age, prevented reproduction in female rats. Rats in which treatment was begun at one or five days had small ovaries with small follicles, atretic follicles and no corpora lutea. Those started at 10 days of age had normal follicles but corpora lutea were inconstant. When treatment was initiated after 10 days of age, the animals recovered. Ovaries were normal with regard to follicles and corpora lutea and pregnancy ensued upon mating with normal males.

In this study a single injection of either 1μg. or 10 μg. of estradiol dipropionate[2] was tested for its effect on reproduction. Swiss albino mice from our colony, maintained on Rockland mouse pellets supplemented by cod liver oil and mazola and lettuce, were injected at 10 days of age. At weaning the treated mice with littermate controls were held together until they were 18 to 25 weeks old. Mating cages were set up with six females, three treated mice with their normal littermates, and one normal male. The results of these matings are recorded in Table 1.

Mice receiving 1 μg of estradiol dipropionate compared favorably with normal littermates in regard to period in mating and size of litters. However in two injected mice parturition did not result until 47 and 49 days after mating.

The majority of mice receiving 10 μg. of estradiol dipropionate were not normal in reproductive response. Only 6 of 15 treated mice produced litters of normal size and nursed them to weaning at 22 days of age, of the remaining 9 animals 2 delivered small litters (3 young) and killed them on the first day of life. Six did not become pregnant during periods in mating of 70 days or more. The remaining animal was found dead after being mated for 20 days and was not pregnant.

All control animals had litters within a normal period of time with one exception (42 days) and all litters were carried through to weaning.

Histological examination of the ovaries of infertile mice revealed numerous atretic follicles but also the presence of from 2 to 4 corpora lutea in each ovary. Adrenal glands contained a ring of brown degeneration pigment at the cortico-medullary junction which has been associated with the senile mouse or mouse under prolonged estrogen treatment. There was no effect on body weight.

Studies are being continued to determine the effect of these two dosages on the reproductive system of both sexes during the first 50 days of life.

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[1] Supported by the Protein Metabolism Fund, Rutgers University.

[2] The estradiol dipropionate was supplied by Ciba Pharmaceutical Products Inc., Summit, New Jersey.

TABLE 1. REPRODUCTIVE RESPONSE OF FEMALE MICE INJECTED WITH ESTRADIOL DIPROPIONATE AT 10 DAYS OF AGE IN COMPARISON WITH LITTERMATE CONTROLS

1 μg. of estradiol dipropionate			10 μg. of estradiol dipropionate		
Age in weeks	Days mated		Age in weeks	Days mated	
	Control	Injected		Control	Injected
18	22	21	20	21	33
	23	24		31	70*
	24	27		33	70*
20	22	22		22	24
	23	23		24	53
	23	27		31	80*
	23	21		42	90*
	24	25	22	23	37†
	28	27		33	80*
22	21	22		23	20‡
	25	47		24	36
25	31	29		26	39
	35	49		29	80*
			24	21	24†
				21	37

All animals delivered litters of normal size and sex distribution except as follows:
* Infertile.
† 3 young killed the first day of life.
‡ Died not pregnant.

SUMMARY

Ten μg. of estradiol dipropionate given as a single injection at 10 days of age caused sterility in 6 of 15 female mice tested with normal males. Two of the mice which delivered young, killed them on the first day of life. The ovaries of sterile mice contained corpora lutea and the adrenals contained brown degeneration pigment. The reproductive ability of female mice receiving 1 μg. of estradiol dipropionate was not affected.

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MEDICAL RESEARCH FELLOWSHIPS

The Division of Medical Sciences of the National Research Council is accepting applications for postdoctoral research fellowships for 1954–1955. These awards are designed to offer research experience for promising individuals who look forward to investigative careers, and not to provide practical experience in the clinical field. Ordinarily Fellowships are not granted to persons over thirty-five years of age. The following programs are announced:

Fellowships in Cancer Research are awarded by the American Cancer Society on recommendation of the Committee on Growth of the Division of Medical Sciences. Awards are available for study in all branches of the biological, chemical and physical sciences and of clinical investigation applicable to the study of growth, typical or malignant. Citizens of the United States are eligible.

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